Identification and molecular analysis of antimicrobial resistant *Salmonella* isolates obtained from pigs slaughtered at Wambizzi abattoir in Uganda

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DECLARATION

I declare that this thesis is my original work and has not been presented elsewhere for a degree award

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DEDICATION

To my late father William Tinega Manyange, my mother Concepta Kerubo Tinega, my self-less wife Mary Nyaituga Osano George and my lovely daughters; Bianca Monica Kemunto George and Barbara Lisa Kerubo George.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACSSuT……Ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline

Amo ……Amoxicillin / clavulanic acid

Amp ……Ampicillin

Cef ……Ceftiofur

Cep ……Cephalothin

Cet ……Ceftriaxone

Cml ……Chloramphenicol

Fox ……Cefoxitin

Gen…… Gentamicinx

Kan ……Kanamycin

MAR……Multi antimicrobial resistance

MDR……Multidrug resistance

MHA……Mueller Hinton agar

MIC……Minimum inhibitory concentrations

PCR……Polymerase chain reaction

QRDR……Quinolone resistance determine region

SGI……Salmonella genomic island

SMR……Small multidrug resistance

Str……Streptomycin

Sul ……Sulfamethoxazol

Tet ……Tetracycline

Tri……Trimethoprim / sulfamethoxazole.

CDC……Centers for Disease Control.

FAO……Food and Agriculture Organization.

WHO……World Health Organization.

ISO……International Standard Organization

CLSI……Clinical Laboratory Standard Institute

MLST……Multi-Locus Sequence Type.

DNA…….Deoxynucleotide acid

RNA…….Ribonucleotide acid

TSB…….Trypticase Soy Broth

BLAST……..Basic Local Alignment Search Tool

www.ncbi.nlm.nih.gov.....National Center for Bioinformation Institution website

ABSTRACT

Non-typhoidal salmonellosis remains an important public health problem worldwide accounting for an estimated 3 million deaths per year. An economic loss of $2.4 billion has been reported in the USA. The emergence of antimicrobial resistance bacteria in both medical and agricultural fields continues to be a serious problem worldwide. Estimates of salmonellosis due to consumption of pork or pork products is difficult to determine but, it ranges from < 1% to 25%. These invasive pathogens colonize intestinal mucosal surface but, they are self-limiting in health individuals due to a noble immunity. This project examined 54 isolates for antimicrobial resistance, sequenced seven housekeeping genes and performed Multi-Locus Sequence Type (MLST) analysis.

β-lactamase and *tetB(B)* genes were detected in 100% and 80% of the isolates respectively. Widespread inter-isolate sequence type (ST) diversity was revealed thereby showing that the sequences types obtained by MLST analysis were genetically divergent. Codon-based Test of Neutrality analysis between sequences revealed *P*-value less than 0.05, an indication of strong forces of natural selection pressure acting at the sequence type level. The Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution discovered frequencies of 0.177 (A), 0.244 (T/U), 0.263 (C), and 0.317 (G). The transition/transversion rate ratios were found to be k_1 = 2.698 (purines) and k_2 = 20.089 (pyrimidines) with and overall transition/transversion bias of R = 6.565, where $R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]$ further confirming that indeed the *Salmonella* isolates studied here were divergent.

These results suggest that, STs coexist in the intestine thereby providing for an efficient intestinal colonization and multiple adaptations. The results also offer general and rapid approaches for identifying genetic diversity of *Salmonella* serotypes in individual pig carcasses which can be adopted for molecular epidemiological surveys of important food contaminating bacterial pathogens.

Based on these results, it will be important to carry out a similar study in Kenya in order to evaluate food safety issues related to consumption of animal source foods particularly pigs. In addition it will be important to initiate a study to identify Single Nucleotide Polymorphisms (SNPs) and consequently contribute to the establishment of a Kenyan bioinformatics database.

Key words; salmonellosis, antimicrobial resistance, isolates, sequence type.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Economic and public health importance of salmonellosis in food animals

Salmonellosis is a food-borne zoonotic disease whose outbreaks are largely due to dramatic changes that occur in the food chain (Scallan *et al*., 2011). There are two types of salmonellosis namely, typhoidal and non-typhoidal salmonellosis (Garcia-Del Portillo, 1999; Ziprin and Hume, 2001). Globally, it is estimated that there are over 21.6 million cases of typhoid *Salmonella* documented annually (Crump and Mintz, 2004). Non-typhoidal *Salmonella* infections are unmistakably common, associated to over 1.3 billion cases with an estimated 3 million deaths annually (Pang *et al*., 1995). Mead *et al*., (1999) also reported estimates of non-typhoidal *Salmonella* infection in the United States of America (USA) to be 1.34 million cases, of which 16,430 persons were hospitalized, and resulting in 553 deaths. The period between 1996 and 1999, in USA, foodborne illnesses from *S.* Typhi alone were estimated to be fewer than 700 cases which resulted to 492 persons being hospitalized with 3 deaths fatalities. Annually, salmonellosis cost US $2.4 billion, resulting from many losses including, medical cost, loss of productivity, and premature deaths (USDA-ERS, 2005). In Denmark, the annual estimated cost of food-borne salmonellosis was estimated to be US$ 15.5 million in 2001 approximately 0.009% of GDP (WHO, 2011). Although, data related to the cost of food-borne salmonellosis are generally not available from developing countries (Okoro *et al*., 2012), salmonellosis which is associated with food animals particularly, pig production is and continues to present serious health problems. *Salmonella* infections represent a considerable burden in both developing and developed countries leading to high medical expense, lost productivity, and mortality (Mead *et al*., 1999; Flint *et al*., 2005; Majowicz *et al.*, 2010).

1.2 The demand for animal source food

There is a continuous demand for livestock food products as a result of population growth (Delgado 2005; USDA-FAS, 2007), urbanization and contributes to a corresponding Gross Domestic Product (GDP) of about 33 percent (CAST, Commentary of May, 2012). Livestock farming occupy about 30 per cent of the terrestrial surface area (FAO, 2006; FAO, 2007) and

are a significant global asset with a value of at least $1.4 trillion. The livestock sector is increasingly organized in long market chains that employ at least 1.3 billion people globally and directly support the livelihoods of 600 million poor smallholder farmers in the developing world (Flint *et al*., 2005; FAO, 2007). The increasing demand for livestock products continues to be a key opportunity for poverty reduction and economic growth (Dijkman, 2009; FAO, 2010). Smallholder livestock farmers are critical to food security for the vast majority of the poor (Kristjanson *et al*., 2004), and this role is not likely to change significantly in the future, particularly in Sub-Saharan Africa (Bruinsma, 2003; Owen *et al*., 2005; Brunori *et al*., 2008; Dijkman, 2009).

1.3 Government regulation and quality control in animal source food

In the face of increasing demand and expanding markets for animal food, there is need for stringent government regulation along animal food distribution chains, including quality control acceptance, guarantee of collective rights and community control (Gura, 2008). Building social protection and strengthening links to urban areas plays a major role in ensuring that livestock farmers are not exploited (Wiggins, 2009; FAO, 2010). There are possibilities that massive investment is taking place in the livestock sector, particularly in developing countries of Sub-Saharan Africa (World Bank, 2009).

1.4 Constraints to livestock productivity in Sub-Saharan Africa

Availability of land, pastures and water have been thought to be the biggest constraints to livestock productivity, however gains have been made through intensification options (Herrero, 2009; Herrero *et al*., 2010). Therefore the biggest constraint to livestock productivity in much of the developing countries in Sub-Saharan Africa is probably livestock diseases (USDA-FAS, 2007; World Bank, 2009). Livestock diseases other than the globally eradicated rinderpest have been associated with a continuous negative impact particularly in Sub-Saharan Africa (Kingsley *et al*., 2009). A good example of such diseases happens to be the host-specific *Salmonella* infections in different animal species within different livestock production systems and which may contribute to conditions which are highly suitable for disease transmission between animals and man (Wallis, 2006). Disease is the most important constraint to Urban Peri-urban Agricultural (UPA) pig farming and production in Kampala (Aliguma, 2004). Death rates estimates amongst pigs have been found to range between 2 and 12 pigs dying per household in 2002 (Aliguma, 2004). African swine fever has been found to be the chief cause of death, followed by accidents and other diseases such as salmonellosis (WHO, 2001; Aliguma, 2004). Disease has been reported to influence reproductive success

or failure thereby disease becomes an insidious cause of production problems (Aliguma, 2004). This increases the cost of pigs relative to slaughter weight by either delaying the days (maturity) to slaughter in the form of more treatment, feed and labour per pig (Aliguma, 2004). (Perry *et al.,* 2002) experts in their report ranked top 10 porcine diseases according to their effect on pig productivity. The rankings showed African swine fever, *Brucella suis*, Cysticercosis, Ectoparasites, Foot and Mouth disease, Helminthosis, Hog Cholera, Japanese B encephalitis, Neonatal mortality and Trypanosomosis. When the diseases were ranked, according to production system, ectoparasites, foot and mouth disease, neonatal mortality, hog cholera and trypanosomosis all were present in the top 20 for UPA. It was however noted, by (Heffernan, 2004) that these rankings did not make use of the voices of the poor and as such did not show the priorities, as seen by the livestock keepers themselves therefore diseases such as pig typhoid was not listed.

1.5 Source of human salmonellosis infections

Salmonellosis infections in humans are linked to either, direct contact with infected livestock or indirectly by the consumption of contaminated animal food or environment. The situation is made worse when compounded with unforeseen use of antimicrobial agents in animal production (Mead *et al.,* 1999). In low income countries *Salmonella* is among the most common bacterial pathogens responsible for as many as one-half of all bacteremia and antimicrobial resistance in man (Prapas *et al*., 2008). The use of sub-therapeutic doses of antimicrobials for growth promotion and nutritional efficiency in pig farming only worsens the situation because antimicrobials evidently selects for resistance (WHO, 2010).

1.6 Antimicrobial resistant salmonellosis

Salmonella serotypes causing human illness are frequently isolated from pigs when a resistant strain is present in an infected individual (Prapas *et al*., 2008; Hendriksen *et al*., 2012). Wherever antimicrobial resistance occurs, treatment failures ensue, the demand for higher antimicrobial doses are administered and this is accompanied by an increased likelihood of long hospitalization (Prapas *et al*., 2008). Apart from the diseases caused by *Salmonella* in the country, the bacteria develop antibiotic resistance resulting to treatment failures with available antimicrobial agents. For example, (Minami *et al.,* 2010) also stated that although the mortality rate caused by salmonellosis decreased significantly in Thailand, the increase of multidrug-resistant strains leads to treatment failure.

1.7 Problem statement

The annual, economic cost associated salmonellosis in the United States was estimated at $2.4billion, resulting from medical cost, loss of productivity, and premature death (US-DAERS, 2005). Non-typhoidal *Salmonella* has been estimated to be responsible for 1.3 billion cases and approximately 3 million deaths annually and it appears to be most prevalent in areas of intensive confinement of livestock (Pang *et al*., 1995). In the United States alone non-typhoidal salmonellosis was estimated to account for 1.34 million cases, of which 16,430 persons were hospitalized and 553 deaths reported (Mead *et al*., 1999 and Harris *et al.*, 2010 Maral Rahmani *et al*., 2013). However, the frightening cost and death estimates are generally lacking for developing countries. Pig farming contributes to a high risk factor for food-borne zoonotic non-typhoidal salmonellosis (Cote *et al*., 2004; USDA-ERS, 2005). The consequence of this is treatment failure and long hospitalization periods (Angulo *et al*., 2004). Several molecular analysis methods have been used in typing and determining the diversity of *Salmonella* isolates. One of such methods and which come cross to satisfying the need for determining sequence types and linking them to antimicrobial resistance, diversity and disease outbreaks (Gantois *et al*., 2009) using housekeeping genes is Multi-Locus Sequence Type (MLST) (Harbottle *et al*., 2006; Achtman *et al*., 2012). Therefore, this study applied conventional, PCR and a novel MLST approach for identification of *Salmonella*, detection of the genes encoding resistance to selected classes of antimicrobial agents used in the treatment of salmonellosis and cluster analysis. This study was the first of its kind to be carried out at Wambizzi pig abattoir in Kampala, Uganda.

1.8 Justification and Significance of this project

Livestock farming is fundamental in public health, social equity and economic growth worldwide (World Bank, 2009). Livestock farming particularly, the small animal rearing sector such as, pigs is critical to food security for the vast majority of the poor in developing countries (World Bank, 2009). In addition, pig farming requires minimal inputs in terms of space, family labour and feeding thus these are the most important motivating factors for farmers to keep pigs (Mutua *et al.*, 2011). Zoonotic food borne salmonellosis has not received the attention it deserves as other re-emerging infectious microorganisms such as *Staphylococci bacteria* and *M. tuberculosis* although salmonellosis is one of the major constraints to livestock production especially the pig sector. Salmonellosis is an exceedingly common and poorly understood zoonotic food borne infection and it is associated with significant adverse sequelae and antimicrobial resistance (Scallan *et al.*, 2011). Given its public health importance, it is

striking that the nature of antimicrobial resistance salmonellosis serotypes of pig origin are not well understood.

1.9 Research questions

1. Is it possible to identify *Salmonella* isolates obtained from pigs slaughtered at Wambizzi abattoir in Kampala, Uganda by using conventional cultural and biochemical methods?

2. Is it possible to identify antimicrobial resistances in *Salmonella* isolates obtained from pigs slaughtered at Wambizzi abattoir in Kampala, Uganda?

3. Is it possible to identify the different serotypes sequence types of *Salmonella* isolates obtained from pigs slaughtered at Wambizzi abattoir in Kampala, Uganda using MLST?

1.10 Null Hypothesis

There is no any genetic diversity between antimicrobial resistant *Salmonella* isolates obtained from pigs slaughtered at Wambizzi pig abattoir in Kampala, Uganda

1.11 Objectives

1.12 General Objective

To identify and characterize antimicrobial resistant *Salmonella* isolates obtained from pigs at Wambizzi pig abattoir in Kampala city in Uganda using conventional culture, biochemical and molecular methods

1.13 Specific Objectives

1. To characterize *Salmonella* isolates obtained from pigs slaughtered at Wambizzi abattoir in Kampala, Uganda using microbial culture and biochemical methods

2. To characterize the identified antimicrobial resistant *Salmonella* isolates obtained from pigs slaughtered at Wambizzi abattoir in Kampala, Uganda using molecular methods

3. To identify sequence types of the identified *Salmonella* isolates obtained from pigs slaughtered at Wambizzi abattoir in Kampala, Uganda using Multilocus Sequence Type (MLST) analysis

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The causative agent of salmonellosis

Salmonellosis is caused by Gram-negative bacteria belonging to the genus *Salmonella* which contains two important species*; S. enterica* and *S. bongori* (Minami *et al*., 2005; Grimont and Weill, 2007). *S. bongori* was formerly subspecies V. Six subspecies are differentiated within *S. enterica* based on their biochemical and genomic characteristics. A roman numeral and a name are used for the designation of these six subspecies as follows: I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae* ; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. Enteric* subsp. *houtenae*, and VI, *S. enterica* subsp. *indica* (Brenner *et al*., 2000; Grimont *et al*., 2003; Grimont and Weill, 2007). *Salmonella* serotypes clearly seem to differ in their pathogenic potential for humans and serotype distributions often vary vastly between human and animal populations. The molecular determinants of serotype-specific and host adaptations have so far largely remained elusive. However, serotype-specific differences in virulence have been characterized in some cases. For instance, in competition experiments *Salmonella* Typhimurium, reptile-associated *S. arizonae* and *S. Diarizonae*, showed a significantly reduced ability for these serotypes to colonize and persist in the intestine of BALB/c mice, clearly suggesting virulence differences (Grimont *et al*., 2003). With regard to food safety *S. enterica* subsp. *enterica* is the subspecies of most concern because the strains within these serogroups are known to cause 99% of *Salmonella* infections in humans (Brenner *et al*., 2000; Grimont *et al*., 2003; Harris *et al*., 2010).

2.2 Descriptive features of *Salmonella* species

Salmonella are facultative anaerobic, Gram-negative, motile small rods (Molbak, 2005). *Salmonella* spp. grow best at the temperature range of between 8°C and 45°C, withstands pH between 4 to 9, and are able to grow at water activities above 0.94. *Salmonella* are heat labile organisms that are inactivated at ordinary cooking temperatures (> 70 °C) although the cooling time and values for temperature and time could change depending on the serotype and the food matrix. In addition *Salmonella* has been shown to tolerate up to 20% salt concentration (Molbak, 2005). *Salmonella* is able to survive for as long as 7 years at temperatures between -23°C to -18°C (Molbak *et al*., 2005). The difficulty in controlling *Salmonella* lies on its ability to survive in extreme environmental conditions (Molbak *et al*., 2005). The biochemical

characteristics of *Salmonella* indicate that, they are able to reduce nitrates to nitrites, produce hydrogen sulphide on Triple Sugar Iron (TSI) agar, their inability to hydrolyze urea to carbondioxide and ammonium hydroxide and their ability to utilize citrate as the sole carbon source (Molbak *et al*., 2005). Salmonellosis infection in man has been implicated to originate from agricultural production due to contamination of food along the food chain (Hendriksen *et al*., 2012).

2.3 Economic and social importance of livestock farming in Kenya and Uganda

The mixed crop–livestock agricultural systems continue to be critical to future food security in many countries because, two-thirds of the global population live in these systems (Kebba and Ofwono, 2007; UBOS, 2008). Mixed farming systems in Africa are facing resource pressures in terms of land, pastures and water availability; however gains have been through intensification options (Herrero, 2009; Herrero *et al*., 2010). Livestock is an important agricultural sub-sector in many developing countries particularly Uganda, where it generates 14 percent of agricultural GDP (Kebba and Ofwono, 2007). Livestock acquisition and intensification is a pathway to poverty alleviation in developing countries (Kristjanson *et al*., 2004). For some poorer households, livestock can provide a means of income diversification to help deal with times of economic stress (Kitalyi *et al*., 2005). Livestock is used for dowry or bride price and as a means to create or strengthen social relationships in developing countries (Kitalyi *et al*., 2005). In East African countries such as Kenya and Uganda, livestock farming including cattle, goat, sheep, pigs and chicken has been growing steadily (Kebba and Ofwono, 2007). The pig farming sector plays an important role in the livelihood of many families in the developing world (Lanada *et al*., 2005). Pig production has a seemingly greater potential to reduce poverty in developing countries (Mutua *et al*., 2011). In many parts of the developing countries such as Kenya and Uganda, pig farming is a very popular economic activity especially with small scale farmers because these farming systems require little space and investment capital (Kagira *et al*., 2010).

The poorest members of most communities in Eastern Africa are often landless, living as squatters consequently they find keeping livestock more adaptable to their situations because livestock occupy small space, are easily moved than crops, and they provide a higher return per unit of land thus offering greater opportunities than other aspects of urban peri-urban agriculture (UPA) (Geundel, 2002). The other benefits accompanying these highly urban, peri-urban adapted farming systems encompass aspects of economics, health and social well-being (Geundel, 2002). Livestock contribute to improved food security through consumption

of high quality protein foods such as meat, milk, eggs and blood (Geundel, 2002). Livestock also can greatly enhance any crop production the household may be engaged in through the use of manure as fertilizer (Geundel, 2002). In other countries of South East Asia for example, aquaculture is also being integrated into livestock-crop systems and therefore providing both food for the household and a high-priced commodity for sale (Geundel, 2002).

Livestock farming is a reliable source of capital especially for women and acts as a living savings bank account which can be accessed throughout the year (Miller, 2001), literally the pig as proverbial 'piggy bank'. Most farmers have developed a strategy of spreading the investment risk within their enterprises in which many of the poorest livestock owners are motivated to keep more than one species (Perry *et al.,* 2002; Kitalyi et al., 2005) with smaller animals such as poultry and pigs offering regular income generation and larger livestock, such as cattle, being utilized to access capital for major expenses such as school fees, development projects or at times of special festivals (Perry *et al.,* 2002).

Excess produce can be sold to supplement household income and in those households where the women are the owner of the livestock this can provide an incredibly important aspect of independence (Perry *et al.,* 2002). Other than the capital incomes, livestock are very important in providing wool and hides, clothing or the ability to build shelters for example in the Masaai communities of Kenya (Perry *et al.,* 2002; Kitalyi *et al*., 2005). Traction such as ploughing in Kenyan and Ugandan livestock rearing communities is solely the work of livestock, such as the oxen while transportation in Asia is via horses or donkeys, which can allow families to get produce to market, or transport family members to schools or hospitals. Socially, livestock keeping are significantly used in some cultures, such as the Masaai of Kenya and the Karamoja of Northern Uganda where large herd sizes are seen as a sign of prestige, often due to the popularity of the use of cattle as 'bride price', where a father may expect up to 100 head of cattle for his daughter ((Perry *et al.,* 2002; Kitalyi *et al*., 2005). In larger community activities such as feasting, weddings, burials, rain-making and other rituals, livestock play important roles (Perry *et al.,* 2002; Kitalyi *et al*., 2005).

2.4 Livestock population in Uganda

The national population of livestock in Uganda as of the year 2008 was estimated at 7.5 Million, with a potential for steady increase due to high demand of meat and as a result of urbanization (UBOS, 2008). Of these, nearly 1.3 million are exotic/cross and the majority (0.9 Million) are in the Western Region of Uganda. The Central Region of Uganda with nearly 2.0 Million has the largest share of indigenous cattle. The total number of chicken is estimated at

20 million, of which 3.7 million (15.7 %) are exotic/cross. The number of goats, sheep and pigs is estimated at 1.1, 1.0 and 2.2 million, respectively. The Central Region leads in pig rearing with 0.8 million (47.1%) of the total livestock. The pig sector is destined to be a major economic activity in Uganda, according to the 2008 estimation of around 3.2 million pigs (UBOS, 2008). However, most infectious diseases such as African swine fever, respiratory and reproductive syndrome, and food-borne zoonotic salmonellosis present a major constraint to pig production (Ojha and Kostrzynska, 2007 and Prapas *et al*., 2008).

2.5 Pig farming in Uganda

Livestock keeping is a major activity in Kampala representing 64% of farming households within different parishes. The most common livestock kept within the range of UPA systems in Kampala are cattle, followed by chicken and pigs (Aliguma, 2004). In a survey by (Napendi *et al.,* 2004) pig production was found to be the most important source of income generation in majority of the parishes apart from Urban Old. Globally, pork has become the most highly consumed meat, comprising 43% of meat consumption (USDA-FAS, 2007). There is an increasing demand for pork in Kampala, with a pig for slaughter reaching a maximum price of UGS 160,000 (Kyaligoza, 2004) with about 76.5% of urban dwellers indicating preference of pork to other types of meats (USDA-FAS, 2007). To meet increasing demand for pork, pig production has become more popular under UPA (Perry *et al.* 2002) with local piglets pricing reaching UGS10,000 per pig (Kyaligoza, 2004). Pigs are known to mature early, produce many offspring and are cheap to feed. There are several benefits associated with pig rearing such as, pork which is either sold or preserved for home consumption and pig manure is used to aid soil fertility (Perry *et al.* 2002). Pig keeping in Uganda is a gender-based activity involving people between 46-60 years, most of whom are women with fenced paddocks or tethering being the preferred method of husbandry (Miller, 2001). Pigs are fed mainly on maize bran purchased from shops (40%) with other feed stuffs such as sweet potatoes leaves, food residues and fish obtained from household leftovers, neighbors and farm produce peelings at the open markets. Some farmers provide additional nutritional supplements in 33% of pigs. Access to veterinary support and drugs is low, but 20% of pig farmers were found to administer de-worming products to their pigs and oil and grease were the most popular anti-tick products applied (Aliguma, 2004).

Urban Peri-urban Agricultural production in pigs affects humans in closest contact to them. There is also a growing concern on environmental contamination, such as water contamination by abattoir effluent, which harbours enteric human pathogens, including, *Cryptospori-*

dium parvum and various species of *Salmonella* bacteria. An average prevalence of salmonellosis in Uganda has been reported to be 28% (UGUS, 2005). Thus, this represents a significant public health risk which may result to numerous outbreaks of gastrointestinal illness due to drinking contaminated drinking water and food products (UGUS, 2005). By their nature most of these infective pathogens are zoonotic diseases of great importance (UN Habitat, 2002) and they include; Bacterial diseases; Anthrax, Brucellosis, Erysipelosis, Leptospyrosis, Tuberclulosis, Salmonellosis and Staphilococosis, Parasitic diseases including Cysticercosis, Trichinellosis, Toxoplasmosis and Sarcoptic mites and mycotic diseases such as dematomycosis. These human health concerns were ranked among most important constraints upon the industry by UPA pig producers in Uruguay enhanced by overcrowding and lack of basic sanitation services (UN Habitat, 2002). Non-typhoidal salmonellosis, a zoonotic disease caused by *Salmonella* species, is thought to originate from livestock farming.

2.6 Salmonellosis in pigs and public health

Pig production contributes to high salmonellosis infections because, the bacterium persists in pigs resulting in asymptomatic 'carrier pigs', which, consequently, generates a major source for *Salmonella* contamination of pork (Bearson *et al*., 2006). Man is infected either by direct contact with pigs and pig manure or by consuming undercooked or ready to eat contaminated pork and pork products (Ojha and Kostrzynska, 2007 and Hendriksen *et al*., 2012). Urbanization stimulates improvements in infrastructure, including cold food storage facilities, and this allows perishable goods such as pork and pork products to be traded more. Salmonellosis is one of the most important zoonotic bacterial diseases of pigs and pork is considered as one of the main sources of human salmonellosis (Hendriksen *et al*., 2012). Worldwide, *Salmonella enterica* subspecies *enterica* serovar Typhimurium (i.e. *Salmonella* Typhimurium) is the predominant serovar isolated from slaughter pigs (Boyen *et al*., 2008). For instance, in 2005, a Typhimurium outbreak among humans in Wisconsin was linked to indirect pig contact in a public setting (Steinmuller *et al*., 2006). Similarly, in 2001 occupational exposure to pigs led to human infection, even though in this case the possibility of a transmission from calves was not conclusively eliminated (Hendriksen *et al*., 2012). *Salmonella* species therefore, represents an occupational hazard for those working with pigs, especially since asymptomatic carriage of broad host-range serotypes appears to be relatively common (Steinmuller *et al*., 2006). Environmental reservoirs plays an important role in maintaining endemic infections, and contaminated clothes have been implicated in the transmission of *Salmonella* from the pigs to man and this indicates the paramount importance of good hygiene practices (Hendrik-

sen *et al*., 2012). An increasing emergence of antimicrobial resistance in enteric pathogens has been on the rise in developing countries (Kariuki, 2010).

2.7 Clinical symptoms in pigs infected with salmonellosis

A variety of clinical symptoms have been observed in *Salmonella* infected pigs, ranging from asymptomatic to acute disease (Cote *et al*., 2004). Infections with serotypes such as Typhimurium usually cause mild or no disease, and infected animals may shed *Salmonella* for considerably long periods of time. For instance, piglets experimentally inoculated with *S.* Typhimurium develop mild gastro-intestinal disease and shed bacteria in their feces for several days (Cote *et al*., 2004). Pig infections with host adapted serotype *S.* Choleraesuis generally causes severe systemic disease with high mortality (Boyen *et al*., 2008). All age groups are susceptible to *Salmonella* infection, but disease is most commonly observed among weaned pigs more than eight weeks of age. Asymptomatic carriers are thought to represent the most important source of *Salmonella* introduction onto pig farms. Other clinical symptoms have been documented among *Salmonella* infected pigs, including enteritis, septicemia, pneumonia, meningitis, and arthritis (Boyen *et al*., 2008). Fever, diarrhea, inappetence, depression, respiratory distress, lameness, edema, and hypoxia in the extremities are common symptoms in clinically sick pigs, and mortality rates in such instances are high (Davis *et al*., 2004). *Salmonella* outbreaks in pigs are manifested as ataxia, fever, depression, diarrhea, and necrotic enteritis, which resulting in approximately 17% mortality (Davis *et al*., 2004).

2.8 Prevalence of salmonellosis in pigs

The prevalence of salmonellosis in pig farms seem to differ considerably by production and management type, on average 12% prevalence has been recorded, but more estimates range from 3.5 to 28% (Davies and Davis, 2010). High *Salmonella* prevalence occurs in large pig breeding farms (Davies *et al*., 2004). However, some studies have reported high prevalence among replacement gilts and finishing gilts, suggesting variability between the herds studied (Davis *et al*. 2004). Higher *Salmonella* prevalence occurs in a highly continuous flow management system, and distinct *Salmonella* serotype populations in breeding herds, nursery and finishing herds from the same farrow-to-finish system have been reported (Davies *et al*., 2004). Breeding herds or nurseries therefore seem to represent epidemiologically important sources of infection in finishing herds, and environmental contamination may play an important role in maintaining endemic infections. Salmonellosis in free finishing herds can lead to endemically infected herds if pigs are strategically moved to clean stalls as they move through the farrow-to-finish system (Crump and Mintz, 2010; Davis and Davis, 2010). Re-

ducing the prevalence of *Salmonella* is particularly important because *Salmonella* prevalence at slaughter tends to be considerably higher than on farm (Hurd *et al*., 2008). Indeed, one study reported 7-fold higher *Salmonella* prevalence in pigs sampled at the abattoir than in animals from the same herds sampled on farm, indicating an important effect of stress or other transportation-related factors (Hurd *et al*., 2008). Host adapted serotype *S.* Choleraesuis, *S.* Typhimurium, *S.* Derby, *S.* Agona and *S.* Anatum are frequently isolated from sick and clinically healthy pigs, indicating a potential risk for human health (Hurd *et al*., 2008).

2.9 Categories of salmonellosis

Two broad categories of salmonellosis have been identified including; non-typhoidal salmonellosis (gastroenteritis) and typhoidal (enteric fever) (Garcia-Del Portillo, 1999; Ziprin and Hume, 2001). *Salmonella* Typhimurium, *S.* Enteritidis, and *S.* Newport are primarily responsible for human and non-human gastroenteritis. Similarly, *S.* Typhi and *S.* Paratyphi serotypes are associated with human enteric fever, while *S.* Choleraesuis is associated with bacteremia in pigs (Ziprin and Hume, 2001). Non-typhoidal salmonellosis has a short incubation period normally 6-8 hours while typhoidal salmonellosis has a longer incubation period ranging from one to ten days. A good example is *S.* Choleraesuis most commonly observed in swine, but nevertheless, the organism is very virulent in humans causing *Salmonella* bacteremia (Gray and Fedorka-Cray, 2002). Enteric fever associated with *S.* Typhi is known as typhoid fever. Enteric fever causes systemic infection and displays symptoms such as fever and abdominal disturbances. In enteric fever, the bacteria migrate from the gastrointestinal tract to the lymphatic system, blood, spleen, and liver resulting in systemic infection (Ziprin and Hume, 2001; Harris *et al*., 2010). The incubation time for typhoid fever is estimated to be between three days and a month. The incubation period for paratyphoid fever ranges from one to ten days (Molbak, *et al*., 2005).

Non-typhoidal *Salmonella* is estimated to be responsible for 1.3 billion cases and approximately 3 million deaths annually and it appears to be most prevalent in areas of intensive confinement of livestock (Pang *et al*., 1995). In the United States alone non-typhoidal salmonellosis was estimated to account for 1.34 million cases, of which 16,430 persons were hospitalized and 553 deaths reported (Mead *et al*., 1999 and Harris *et al.,* 2010). The annual, economic cost of salmonellosis in the United States is estimated at $2.4billion, resulting from medical cost, loss of productivity, and premature death (USDAERS, 2005). Reported cases of non-typhoidal salmonellosis indicates that there were approximately 28,000 cases of non-

typhoidal *Salmonella* which resulted to 5000 patients being hospitalized (Helms *et al*., 2006) in Denmark, from 1991 to 2000. Unlike in industrialized countries where *Salmonella* surveillance and monitoring systems are implemented to track outbreaks, institute control measures and identifies the serotypes involved (Olsen *et al*., 2001), there are limited *Salmonella* data available in less developed countries in Africa, because these countries lack funding, proper testing laboratories, and personnel to adequately isolate and characterize the organism (Crump and Mintz, 2004).

According to CDC, (2005), approximately 99% of all human salmonellosis is attributed to *S. enterica* subsp. *Enterica*. The naming of *Salmonella* serotypes is based on the disease caused or animal species from which the bacterium was first recovered or isolated. For example in human medicine, serotype names are linked to the bacteria that cause the infection, such as *S.* Paratyphi A and *S.* typhi. Similarly, serotypes including, *S.* bovis in cattle, *S.* Abortusovis in sheep, *S.* Gallinarum in poultry and *S.* Choleraesuis in pigs (Grimont *et al*., 2003). Other serotypes are named according to the geographical location where the pathogen was isolated, thus serotypes such as *S.* Dublin, *S.* Panama, *S.* Paul and *S.* Heidelberg (Anderson and Ziprin, 2001). With reference to naming, only the genus is italicized and the first letter of the serotype is capitalized. For example, *Salmonella* enterica subsp. *Enterica* serotype *Typhimurium* is now written as *Salmonella enterica* subsp. *enterica* Typhimurium or for simplicity *S.* Typhimurium (Brenner *et al*., 2000 and Andrews and Baumler, 2005)

In addition to the naming system, host adaptation is used to classify *Salmonella* into three other categories including those highly adapted to man such as *S.* Typhi, *S.* Paratyphi A, *S.* Paratyphi B, and *S.* Paratyphi C (Grimont *et al*., 2003). 1). The serotypes which are the causative agents of typhoid fever, 2) those highly adapted to specific non-human hosts (intermediate) such as *S.* Abortusovis in sheep, *S.* Dublin in cattle, *S.* Choleraesuis, in pigs (swine) and *S.* Pullorum or *S*. Gallinarium in poultry (Grimont *et al*., 2003). Serotypes in this group rarely cause human illness and 3) those un-adapted to specific hosts because they are ubiquitous and they are responsible for dramatic salmonellosis (Wallis, 2006). Though salmonellosis can affect all species of animals including man where they are linked to specific clinical syndromes (Gray and Fedorka-Cray, 2002), domestic animals, young animals, the pregnant and lactating animals are thought to be most susceptible (Chu and Chu, 2006). Published reports (Molbak *et al*., 2005) indicate that, *S.* Typhimurium, *S.* Enteritidis, and *S.* Newport are primarily responsible for human and non-human gastroenteritis. Similarly, *S.* Typhi and *S*. Para-

typhi serotypes are associated with human enteric fever, whereas *S.* Choleraesuis is associated bacteremia in pigs (Gray and Fedorka-Cray, 2002).

2.10 Non-typhoidal salmonellosis

Non-typhoidal *Salmonella* belonging to different serotypes represents one of the most important foodborne pathogens causing gastroenteritis in man. Non-typhoidal *Salmonella enterica* strains such as, *S.* Typhimurium, *S.* Enteritidis, and *S.* Newport which cause human and non-human gastroenteritis are easily passed from animals to humans and are thus classified as zoonotic pathogens(Gray and Fedorka-Cray 2002). They can colonize or infect humans as well as a variety of domesticated and wild animals ranging from mammals to birds and reptiles (Carattoli, 2003). Gastroenteritis infections caused by non-typhoidal *Salmonella* are mostly self-limiting and treatment is not required. However, approximately 5% of human individuals with gastrointestinal illness will develop bacteremia, which is a serious and potentially fatal problem that requires treatment interventions. The currently recommended drugs of choice for treatment of salmonellosis are fluoroquinolones and third-generation or extended-spectrum cephalosporins (Carattoli, 2003).

The risk presented by *Salmonella* serotypes such as foodborne hazards, are accompanied by rapidly emerging multidrug-resistant *Salmonella*. This can be attributed to the widespread use of many antimicrobials in human and veterinary medicine, such as sulphonamides, trimethoprim, aminoglycosides, chloramphenicol and tetracycline promoting development of drug resistance leading to serious public health concerns (Su *et al*., 2004). *Salmonella enterica* strains belonging to different serotypes and showing resistance to four or more antimicrobials, including fluoroquinolones and third-generation cephalosporins, are widespread (Skold, 2001). Most of these strains are zoonotic possibly acquiring resistance from food-animal hosts and causing human infections along the food chain (Parry and Threlfall, 2008). The increasing resistance seen in *Salmonella* not only complicates and limits treatment options in affected animals and humans but has also been shown to increase the active transfer of antimicrobial resistance (AMR) determinants to other microbes (Rayamajhi *et al*., 2008). Variations in resistance in circulating *Salmonella* serotypes express different genes coding for resistance towards a variety of antimicrobial agents such as β-lactam and tetracycline antimicrobial agents. This results in higher minimum inhibition concentrations (MICs) although inter and intra-variations occur in different *Salmonella* isolates. Horizontal transfer of genes coding for resistance in *Salmonella* serotypes present increasing opportunities for resistance

genes to be transferred and maintained in the population (van and Schaik Willems, 2010; Zhou *et al*., 2013). Such resistance is difficulty to determine and continue to be disseminated among bacteria, animal source food (Wary and Gnanou, 2000) and eventually to man leading to the cultures of "super burg" *Salmonella*, which are difficult to manage with available antimicrobial agents (Botteldoorn *et al*., 2003).

In human, salmonellosis is considered as one of the most common and economically important zoonotic diseases. Intestinal salmonellae are widespread in the environment: - commonly found in farm effluents, human sewage and in any material subject to fecal contamination. *Salmonella* organisms may also be found in animal feedstuffs, causing subclinical gastrointestinal carriage or infectious disease in animals, particularly poultry and pigs (Wray and Gnanou, 2000; Molbak *et al*., 2005).Consumption of animal source foods has been linked to antimicrobial resistance observed in management of salmonellosis in humans (Wray and Gnanou, 2000; Molla *et al*., 2003; Grimont and Weill, 2007).

2.11 Typhoidal salmonellosis

Typhoid fever, sometimes known as enteric fever, is a disease caused by the bacterium *Salmonella enterica* serotype Typhi. Typhoid fever is a serious disease which can be life threatening unless treated promptly with appropriate antimicrobials (Bywater *et al.,* 2004). The treatment may even be instituted before the results of laboratory sensitivity tests are available. The severity of typhoid fever varies greatly, but nearly all patients experience fever and headache. Slightly less serious, but nevertheless very debilitating and possibly fatal, is enteric fever resulting from infections with *Salmonella* Paratyphi A (Garcia-Del Portillo, 1999; Brenner *et al*., 2000). Again, appropriate antimicrobial treatment is essential and should be commenced as soon as the disease is diagnosed.

2.12 Treatment of human salmonellosis

The first-line antimicrobials of choice for the treatment of both *Salmonella* Typhi and *S.* paratyphi A are fluoroquinolones such as ciprofloxacin, with third generation cephalosporins and azithromycin considered as possible alternatives (Bhutta, 2006). *Salmonella* Typhi and *S.* paratyphi A are infections linked to travel to endemic areas such as the Indian sub-continent, Africa, or south and Central America (USDA-ERS, 2005). Antimicrobial resistance to therapeutically important antimicrobials is one of the major concerns, and strains with resistance or decreased susceptibility to antimicrobials such as ciprofloxacin are becoming widespread

in developing countries, such as Kenya and Uganda and consequently in travelers returning from these areas (Threlfall, 2002).

2.13 Antimicrobial uses in pig production and the emergence of antimicrobial resistance *Salmonella* serotypes

The use of antimicrobial agents in agricultural production has been seen as a major driver of antimicrobial resistance in pathogenic bacteria of significance to human (Angulo *et al*., 2004). There are three ways in which antimicrobial drugs are used in animals including therapy, prophylaxis, and growth promotion (Tollefson and Miller, 2000). Therapeutic use of antimicrobial agents is intended to cure an existing bacterial infection and group treatment of food-producing animals is administered because individual animal treatment is often impractical (Tollefson and Miller, 2000). Prophylaxis is carried out to prevent infections and maintain a health herd of a group of animals. Such a practice is thought to reduce the numbers of sick and consequently death of animals thereby decreasing the amount of antimicrobial agents needed to treat large numbers of the symptomatically ill animals thus, reducing treatment costs. In pig production, antimicrobials are prophylactically used at different stages of growth, such as weaning and when a new pig or piglet has been bought to increase the herd or before mixing of pigs from different herds (Tollefson and miller, 2000). Problems associated with drug residues in beef resulting from feeds and therapy cannot be overemphasized (Kaneene and Miller, 1997; Cromwell, 2002). Although prophylactic herd treatment is criticized for its role on the selection of resistance among pathogenic bacteria (van den Bogaard *et al*., 2000), antimicrobial prophylaxis at these key periods for disease incidence is an unavoidable measure in the pork producing systems. Even more critically assessed is the use of antimicrobial agents for growth promotion in food animals (van den Bogaard *et al*., 2000). The mode of action of antimicrobial growth promoters is not fully understood. However, the main effects are believed to be a reduction of the growth of bacteria in the intestinal tract and thereby less microbial degradation of useful nutrients, and the prevention of infections with pathogenic bacteria (Aarestrup, 1999). Numerous studies on the economic benefit of the use of antimicrobial growth promoters have been performed. In general, an improvement in growth rate and feed utilization has been observed. To increase pig production and productivity, farmers use antimicrobial drugs for nutritional efficiency (van den Bogaard *et al*., 2000). However there has been a growing concern because the use of sub-therapeutic doses of antimicrobials for prophylaxis in agricultural animals particularly pigs is leading to selection of resistant bacterial strains such as *Salmonella* (Aarestrup *et al*., 2003). The use of sub-

therapeutic doses of antimicrobials for growth promotion and nutritional efficiency in pig farming only worsens the situation (WHO, 2007). The prophylactic use of antimicrobial agents in animal populations has been of a particular concern (Aarestrup, 2003) especially when the antimicrobial classes used in pigs are the same as, or related to, the pharmaceuticals used in the control of human infections because exposure of microbial populations to antimicrobial agents evidently selects for resistance gene determinants (Varma *et al*., 2007). *Salmonella* serotypes causing human illness are frequently isolated from pigs and when a resistant strain is present in an infected individual, medical treatment effectiveness decreases resulting in the need for higher doses and increased hospitalization periods of time (Prapas *et al*., 2008).

2.14 Origins and consequence of antimicrobial resistance in food borne pathogens

The use of antimicrobials in human and animal since the discovery of penicillin in the late 1920s dramatically reduced the morbidity and mortality associated with numerous infectious diseases. This also resulted in an unprecedented global increase in the incidence of clinical bacterial strains that are multiple resistant to antibiotics. Bacterial antimicrobial resistance in both the medical and agricultural fields has become a serious problem worldwide (McDermott *et al*., 2002). Antibiotic resistant strains of bacteria are an increasing threat to animal and human health, with resistance mechanisms having been identified and described for all known antimicrobials currently available for clinical use. The administration of therapeutic and sub-therapeutic antimicrobials to animals causes the emergence and dissemination of multiple-antibiotic-resistant zoonotic bacterial pathogens, which has become an area of increased public health and scientific interest (Mellon *et al*., 2001).Research has linked the use of antibiotics in agriculture to the emergence of antibiotic resistant foodborne pathogens (Menezes *et al*., 2010). Bacterial pathogens of animal and human origin are becoming increasingly resistant to most front line antimicrobials, including broad-spectrum cephalosporins, aminoglycosides, and fluoroquinolones. Increased incidence of antimicrobial resistant *Salmonella* for example, has severe implications for the future treatment and prevention of infectious diseases in animals and humans (Menezes *et al*., 2010).

2.15 The association between the use of antimicrobials in food animals and the occurrence of antimicrobial resistance

A substantial portion of antimicrobial agents is used for growth promotion and prophylactic purposes (Mellon *et al*., 2001). Thus, bacterial species related to food animals are exposed to a substantial constant natural selection pressure. This selective pressure favors the emergence

of antimicrobial resistance in zoonotic pathogens such as *Campylobacter, Yersinia, Listeria* and *diarrheagenic E. coli* and *Salmonella,* which are frequently harbored in the animal intestinal tract. It also favors the selection of antimicrobial resistance genes in non-pathogenic bacteria (Aarestrup, 1999), which later may transfer the acquired resistance to different pathogenic bacterial species leading to variant *Salmonella* genomic island (Doublet *et al*., 2004). Knowledge regarding associations between use of antimicrobial agents and occurrence of resistance is in many cases based on experience of changes in occurrence of resistance in relation to introduction of new agents. There are some good examples of the introduction of antimicrobials for animal use and the emergence of resistant bacteria resulting to genetic divergence (Liu *et al*., 2009). Fluoroquinolone resistance among *S. Typhimurium* DT204 has been documented after the introduction of cenrofloxacin for veterinary use in 1989 (Hendriksen *et al*., 2012). Aminoglycoside antibiotic apramycin introduction for veterinary use at the beginning of the 1980s, lead to the emergency of resistance to apramycin among *E. coli* isolates found in cattle and pigs in France and the UK. Although apramycin has never been used for treatment of infections in humans, the gene *aac(3)-IV* encoding resistance to apramycin also confers resistance to gentamicin, which is widely used in humans (Botteldoorn *et al*., 2004). The same resistance gene and plasmid have since been found in Salmonella from animals and in human clinical *E. coli* isolates (Botteldoorn *et al*., 2004). These observations strongly suggest that this resistance gene primarily emerged among food animals because of the selection by use of aparamycin in food animals, and was then transmitted to humans. A possible explanation for this observation is that ceftriaxone-resistant *Salmonella* in pork meats have arisen due to cross-resistance between ceftriaxone and ceftiofur, a cephalosporin used in food animals (Winokur *et al*., 2000). Simply showing that a growing proportion of pathogens and commensal organisms isolated from food animals are resistant to antimicrobial agents is not enough to prove a human health hazard. Rather, it must be demonstrated that as a result of such antimicrobial resistance infections are more numerous or are more severe or are less easily treated than would be the case otherwise. There are some potential mechanisms of increased disease in humans from antimicrobial resistance in food animals (Travers and Barza, 2002). First, exposure of food animals to antimicrobial agents can not only select antimicrobial-resistant pathogens, but also might lead to increased colonization of the animals by antimicrobial-resistant pathogens (Travers and Barza, 2002). This is because treatment with antimicrobial agents often results in the reduction of various components of the commensal flora, which normally exerts a protective effect against colonization and infection of exogenous organisms (Travers and Barza, 2002). This increase in the number of pathogens in

food animals could lead to an increase in the burden of pathogens in the environment and in the food chain up to human consumers (Travers and Barza, 2002). Furthermore, as in humans, most of these pathogens would presumably be resistant to antimicrobial agents. Second, antimicrobial resistance arising in food animals could involve not just obvious pathogens but relatively nonpathogenic bacteria such as generic *E. coli* or *Enterococcus species* (Van Schaik and Willems, 2010). These organisms could become reservoirs of mobile antimicrobial resistance elements that could colonize humans via food or environmental exposure (Van den Bogaard *et al*., 2000). These resistance elements could then be transferred to pathogens in the gut (Van den Bogaard *et al*., 2000). Third, infection by antimicrobial-resistant microorganisms might have a worse outcome due to ineffective initial treatment, the need to use less desirable treatment options, or both (Van den Bogaard *et al*., 2000). The initial empiric treatment choice might be an antimicrobial agent to which the pathogen is resistant, leading to a delay in effective therapy (Olsen *et al*., 2001). Effective agents might be more toxic, more expensive, or more difficult to administer than traditional choices (Olsen *et al*., 2001).

2.16 Mechanisms of antimicrobial resistance

Resistance to antimicrobial agents in bacteria is mediated by several mechanisms, including the occurrence of integrons and resistance genes among sulphonamide-resistant leading to 1) changes of bacterial cell wall permeability, 2) energy-dependent removal of antimicrobials via membrane bound efflux pumps, 3) modification of the site of drug action, and 4) destruction or inactivation of antimicrobials (Peirano *et al*., 2005). Acquired antimicrobial resistance phenotypes most often develop via conjugative transfer of plasmids and the occurrence of operons that cause alteration of resistance (Rodas *et al*., 2010). Plasmids may carry class I integrons, mobile DNA elements, which are consequently important in the proliferation and dissemination of bacterial multidrug resistance, especially among the Gram-negative enteric species (Verdet *et al*., 2000 and Winokur *et al*., 2001). Integrons have been primarily found located within transposons such as Tn402 and Tn21, which are also found residing on broad-host-range plasmids or the lncF plasmid (Villa *et al*., 2000; Aerestrup *et al*., 2003). Integrons location which incorporates transposons and plasmids enhances their ability to capture and dissemination of resistance genes among bacteria.

2.17 Antimicrobial resistance in *Salmonella*

The antimicrobial resistance in bacteria has shown a steady increase in recent years. At least 17 classes of antimicrobial agents are approved for growth promotion and feed efficiency in livestock including: tetracyclines, penicillins, macrolides, lincomycin, and virginiamycin.

The use of antimicrobial agents in food animals creates a selective pressure for the emergence and dissemination of antimicrobial-resistant bacteria including animal pathogens, human pathogens which have food animal reservoirs, and other bacteria which are present in food animals (Van den Bogaard *et al*., 2000). *S.* Typhimurium is frequently associated with multidrug resistance (Rodas *et al*., 2010) in part due to the worldwide emergence of *S.* Typhimurium definitive phage type (DT) 104, which contains the chromosomal *Salmonella* genomic island type I (SGI-1). The origin of resistance in *Salmonella* is thought to be SGI-1, which harbors genes that confer the ACSSuT phenotype which, means that, this phenotype is resistance to five antibiotics in a combination of ampicillin, chloramphenicol, streptomycin, sul-fonamides, and tetracycline (Angulo *et al*., 2000). Although *S.* Typhimurium DT104 is the main example of multi-resistance in *S. enterica,* many antimicrobial resistance genes have been reported also in isolates of other serotypes (Hamidian *et al.,* 2009; Morshed and Peighambari, 2010). SGI1 harbors a cluster of genes containing the complex class 1 integron that encodes multidrug resistance, most often associated with the ACSSuT pentaresistance to amoxicillin ($^{bla}_{\text{PSE-1}}$), chloramphenicol/florfenicol (*floR*), streptomycin/spectinomycin (*aadA2*), sulfonamide (*sul1*) and tetracycline (*tetG*) (Zankari *et al*., 2012). Antimicrobial resistance to beta-lactams has also been reported in isolates from human and animal sources (Hamidian *et al*., 2009; Morshed and Peighambari, 2010). Resistance mechanisms such as penicillinase hyperproduction, extended spectrum beta-lactamases (ESBL) (Madec *et al*., 2011) or inhibitor-resistant ^{bla}*CTX-M2* beta-lactamase are encoded by the plasmid-mediated ^{bla}*CTX-M2* gene (Madec *et al*., 2011). The presence and dissemination of ^{bla}*CTX-M2* genes are a serious public health issue, and could be responsible of treatment failure (Hamidian *et al*., 2009; Morshed and Peighambari, 2010). Molecular genetic techniques have been used to characterize antimicrobial resistant *Salmonella*e, especially *S.* Typhimurium DT104 (Rodas *et al*., 2010). For instance, a variant *Salmonella* genomic island 1 (SGI1), multidrug resistance (MDR) regions, consisting of integrons encoding different resistance genes, located in the chromosomal DNA of *S.* Typhimurium DT104 and Agona was reported by (Boyd, 2002). The formation of these multidrug resistance clusters favors the expression of a large number of resistance genes and enhances their transfer to other bacteria. According to (Cloeckaert, 2000; Boyd, 2002), class I integrons have been found to be integrated into the chromosome of *S.* Typhimurium DT104 and Agona, thereby becoming more persistent even in the absence of antimicrobial selection and with no apparent fitness cost to bacterial cell.

2.18 Review of the techniques used for isolation and identification *Salmonella* from pork and fecal samples

2.18.1 Isolation of *Salmonella* from samples

Isolation typically involves pre-enrichment of 25g of samples in non-selective medium so as to recover sub-lethally injured cells due to heat, cold, acid, or osmotic shock (Codex Alimentarius Commission, 2007). Specifically, the samples are homogenized using autoclave-sterilized cold Buffered Peptone Water (BPW, Difco, UK) which is a non-selective broth media (Kang and Fung 200) and incubated at 37^0C for 24 hours (Codex Alimentarius Commission, 2007). The main aim here is to increase target *Salmonella* bacterial cells which are normally not uniformly distributed in the samples but typically occur in low numbers, and may be present in a mixed microbial population (Codex Alimentarius Commission, 2007). Here 1ml of the primary enriched 'bug' cultures is transferred into tetrathionate enrichment (TT) broth medium in a 10ml vial and this secondary selective medium added to make a 5ml final volume. The cultures are incubated at 37°C for 24 hours and struck onto Xylose Lysine Deoxycholate selective medium (XLD difco, UK) agar plates (Kang and Fung 2000; Kim and Bhunia 2008). The plates are observed for bacterial colony forming units. Typical *Salmonella* colonies have a round smooth morphology with black/pink centers on selective XLD agar (Kim and Bhunia 2008).

2.18.2 Review of biochemical tests used to identify *Salmonella* isolates

2.18.2.1 Indole test

Hydrolysis of Indole by *Salmonella* leads to the formation of a pinkish ring at the surface of the broth medium in the test tube. *Salmonella* produces the enzyme needed to catalyze the breakdown of Indole into its constituent components during incubation at 37^0C over night (Anderson and Ziprin, 2001). Indole is generated by reductive deamination from tryptophan via the intermediate molecule Indolepyruvic acid. Tryptophanase catalyzes the deamination reaction, during which the amine ($-NH_2$) group of the tryptophan molecule is removed. Final products of the reaction are indole, pyruvic acid, ammonium (NH_4) and energy. Pyridoxal phosphate is required as a coenzyme (Finazzo and Obenauf, 2009). $C_{11}H_{12}N_2O_2 + H_2O \xrightarrow{\text{tryptophanse}} C_8H_7N + C_3H_4O_3 + NH_3$

2.18.2.2 Triple Iron Sugar test (TSI) for hydrogen sulfide production

Salmonella has the ability to digest (enzymatically) the sugars such as glucose and/or lactose leading to the production of hydrogen sulfide (H_2S) (Anderson and Ziprin, 2001). *Salmonella*

produces an enzyme which aids in the enzymatic break down of sugars and allows bacteria to produce ATP under oxygen limiting conditions, producing acids and/or gas (CO_2) as a byproduct (Finazzo and Obenauf, 2009). Glucose is the first sugar to be catabolized. If glucose is unavailable or used up, a bacterium reaches utilizes an alternative pathways but, requiring more energy expenditure (Finazzo and Obenauf, 2009). The organism produces the enzymes to hydrolyze the disaccharide lactose, the organism and continues fermenting to assist in ATP generation. However, if the glucose is limiting and the organism does not produce the necessary enzymes to catabolize lactose, the organism can utilize the protein in the medium via the process of deamination. Normally, the medium has sodium thiosulfate ($Na_2S_2O_3$) as a major component and it is sodium thiosulfate which undergoes enzymatic breakdown in several stages. The H_2S produced is the one responsible for the blackening of the media since the medium contains a pH indicator for H_2S(Anderson and Ziprin, 2001). Na-S-S + S-SO_3- $\longrightarrow$ Na-S-S-S-SO_3-

Na-S-S-S-SO_3- + 2 glutathione $\longrightarrow$ Na-S-S + oxidized glutathione + H_2S + $S0_3$-

2.18.2.3 Simon Citrate test

Salmonella has the ability to utilize citrate as a sole carbon source. When inoculated on a medium containing Simon citrate *Salmonella* utilize citrates as the sole source of energy and releases bromothymol blue which in the presence of a pH indicator turns the medium from green to deep blue. Use of citrate involves the enzyme citrase, which breaks down citrate to oxaloacetate and acetate (CLSI, 2010). Oxaloacetate is further broken down to pyruvate and carbon dioxide (CO_2). Production of sodium bicarbonate ($NaHCO_3$) as well as ammonia (NH_3) from the use of sodium citrate and ammonium salts results in alkaline pH (Anderson and Ziprin, 2001). $C_3H_5O(COO)_3^{3-} \xrightarrow{\text{citrase enzyme}} {}^{-}O_2CCH_2C(O)CO_2^{-} + [CH_3COO]^{-}$

${}^{-}O_2CCH_2C(O)CO_2^{-} \xrightarrow{\text{pyruvate carboxylase}} CH_3COCOO^{-} + CO_2$.

$CH_3COCOO^{-} + CO_2 + Na^{+} + H_2O + NH^{-4} \longrightarrow NaHCO_3 + NH_3 + [CH_3COO]^{-}$.

2.18.2.4 Urease negative confirmatory test

Salmonella is unable to hydrolyze urea. Urease is an enzyme that catalyzes the hydrolysis of urea to unstable carbamic acid. Rapid decomposition or hydrolysis of carbamic acid (ammonia rich) occurs without enzyme catalysis to form ammonia and carbon dioxide (Benini *et al.,* 1999). The ammonia produced reacts with water to form ammonium ions (NH_4^{+}), or the gas definitely escapes to the atmosphere. CO (NH2)2 + $H_2O \xrightarrow{\text{Urease enzyme}} NH_3^{+}$ CO_2. This test is used as a confirmatory test for *Salmonella* thereby distinguishing *Salmonella*

from *Proteus* species which like, *Salmonella*, also forms black colonies in Xylose deoxycholate (XLD) agar. *Proteus* species produces an enzyme which catalyzes the hydrolysis of urea (Benini *et al*., 1999).

2.19 Review of molecular analysis methods used in identification of *Salmonella* isolates

Historically, conventional methods of pathogen detection have been techniques of choice, but more recently, testing laboratories are utilizing genomic DNA (Wilson, 2001) PCR-based (Wilson *et al*., 1990; Johnson and Clabots, 2000) methods for this task. Phenotypic methods of testing are known to show poor reproducibility, low sensitivity, are labor intensive, expensive, and slow in pathogen identification (Anderson and Ziprin, 2001). Molecular DNA analysis technologies enable scientists to identify and detect novel and previously uncharacterized microorganisms. Conventional culture methods therefore have been supplanted by these nucleic acid amplification technology (Rys and Persing, 1993) and *in situ* oligonucleotide hybridization (Amann *et al*., 1995) for ‘‘growing’’ and ‘‘seeing’’ some microorganisms. The power of these techniques has opened a new chapter on the diversity of environmental, human and animal associated microorganisms (Wilson *et al*., 1990; Urwin and Maiden, 2003). PCR-based molecular techniques have been widely accepted as an alternative to conventional methods in pathogens detection (Jitrapakdee *et al*., 1995; Johnson and Clabots, 2000). Techniques such as denaturing gradient gel electrophoresis (DGGE), pulsed field gel electrophoresis (PFGE), repetitive extragenic palindromic-PCR (REPPCR), real-time PCR (Harbottle *et al*., 2006) and multi-locus sequence type (Achtman *et al*., 2012) are commonly used to discriminate among bacterial species, serotypes, and strains. The current study focused on the use of MLST and PCR sequencing in identifying *Salmonella* serotypes. MLST (Urwin and Maiden, 2003) targets the conserved housekeeping genes sequences. MLST exploits the genomic DNA fragments sequences and the protocol has previously been described by (Wilson, 2001; Urwin and Maiden, 2003; Achtman *et al*., 2012) for foodborne pathogens identification. These sequences are found among all cellular life and reliably predict organismal phylogeny (Woese, 1987). MLST provides highly specific information about the evolutionary history of a microbial pathogen and hence its taxonomic position relative to those of other known organisms is determined by cluster analysis (Aanensen and Spratt 2005; Achtman *et al*., 2012). The prevalence, characteristics and distribution of non-typhoidal *Salmonella* in diarrhea patients and slaughter animals in Uganda districts have been previously reported by (Nasinyama *et al*., 1998). However, occurrence and spread of antimicrobial resistance and specific sequence types (STs) of non-typhoidal

Salmonella in pigs slaughtered at the Wambizzi abattoir, which handles pigs from different parts of Uganda, has never been reported. It was therefore important to undertake research to characterize circulating strains in pigs, compare them with known *Salmonella* Typhimurium isolate sequence obtained from publicly accessible database at www.ncbi.nlm.nih.gov and explore opportunities to combat the spread of non-typhoidal salmonellosis through the food chain to humans.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study site

The study was undertaken in three main sites including Wambizzi abattoir in Kampala, Uganda, Makerere University in Kampala, Uganda and Bioscience Eastern and Central Africa (BeCA)-Hub at the International Livestock Research Institute (ILRI) in Nairobi, Kenya.

The samples were collected from pigs slaughtered at the Wambizzi abattoir which is a Farmers' Cooperative Society. The Society was founded in 1971, and remains the only registered pig abattoir in Kampala and serves as an important part of the pig value chain. The abattoir promotes piggery and facilitates transport to the slaughterhouse and provides for market access in urban and peri-urban areas in Kampala, Uganda. The abattoir supplies Kampala city with pork. According to the abattoir administrators, it has an operating capacity of roughly 200 pig slaughters per day but, the delivery of pigs to the abattoir is largely erratic. Some of the problems faced by pig farmers in Uganda include diarrhea diseases, low prices (buyers consider the weight of the carcasses rather than the live weight of pigs) and exploitation by middlemen (Napendi *et al.,* 2004).

Conventional culture methods, biochemical tests and antimicrobial susceptibility tests were performed at Makerere University in Kampala, so as to isolate and identify antimicrobial resistant *Salmonella*. Molecular and bioinformatics analysis were performed at the Bioscience Eastern and Central Africa (BeCa)-Hub, molecular biology laboratories within the International Livestock Research Institute (ILRI) in Nairobi, Kenya. Here the genes coding for antimicrobial resistance to selected agents were evaluated using specific primers. Further analysis by Multilocus sequence type (MLST) was performed to identify the isolates sequence types (STs). Finally, based on the MLST sequence types (STs) a taxonomic cluster analysis was performed and an, overall phylogenetic relationships constructed.

3.2 Determination of the sample size

The sample size was determined according to Alonzo *et al.*, (2002) using a binomial one-sample calculation formula as outlined below.

$$n = \frac{\left(Z_{\frac{\alpha}{2}} \sqrt{p(1-p)} \right)^2}{d^2}$$

$Z_{\alpha/2}$ = 1.96, the level of significance required for estimating the prevalence of *Salmonella* (Z-normal value for 95% confidence, or equivalently 5% significance is 1.96), the margin of error (d) = 6% and the assumption for *Salmonella* levels, using a previous survey of pig salmonellosis infection rate (Nasinyama *et al*., 1998) is expected to be (p) = 9%. The calculated sample size was n=162. However, considering the unforeseen and likely sampling problems, the actual sample size was reduced to 100 (100 fecal and 100 muscle samples). Similar sample sizes have been reported elsewhere (Nasinyama *et al*., 1998) therefore this sample size did not negatively affect the attainment of the objectives of this study.

3.3 Sample collection

All the pig carcasses were identified using edible ink in order to prevent poisoning or toxicity and samples from 100 animals were pooled. A total of 100 gluteal muscle samples were taken using sterile knives and transferred into sterile plastic caps containing Stuart transportation medium (Oxoid Limited Wade Road Basingstoke Hampshire RG24 8PW, United Kingdom) which was used to preserve the samples and maintain viability of bacteria. Similarly, 100 fecal samples were taken from the carcasses after opening the ileo-ceacal junction of the intestines and drained into sterile plastic caps containing Stuart transport medium. The samples were transported to the Applied Food Science Laboratory, College of Veterinary Medicine, Animal Resources and Biosecurity (COVAB), at Makerere University where they were kept at -80^{0}C until further analysis.

3.4 Media and sample preparation

3.4.1 Buffered Peptone water (BPW)

Peptone water (Thermo Scientific, Oxoid microbiology products, UK) was prepared according to the manufacturer's instructions by dissolving 25g of peptone powder in 1liters of distilled water. This was followed by heat sterilization by autoclaving at 121^0C for 1 hour. Autoclaved peptone water was allowed to cool at room temperature, and aseptically dispensed into sterile 225 ml flat bottomed flasks for later use in pre-enrichment of the samples for *Salmonella* culturing and isolation.

3.4.2 Preparation of tetrathionate broth for enrichment

Tetrathionate broth media (Thermo Scientific, Oxoid microbiology products, UK) was prepared following the manufacturers instruction by weighing 6 grams of tetrathionate powder and dissolving it in sterile 1000 ml distilled water. The solvent was heated on a hot plate with frequent agitation until the medium boiled and dissolved completely. The solvent was cooled at room temperature to around 55°C. Meanwhile, 20 ml Iodine solution containing 5g potassium iodide and 6g of iodine crystals was prepared and added to the tetrathionate broth. The addition of Iodine solution is important in order to activate the bacterial cells which might have been inactivated by long periods of deep freezing.

3.4.3 Preparation of XLD medium

55.2 g of dehydrated medium (Thermo Scientific, Oxoid microbiology products, UK) were suspended in 1 liter of deionized water and heated to approximately 100°C using a hot plate with frequent agitation and heating was stopped when the medium boiled and was completely dissolved. The medium was allowed to cool at room temperature and its temperature maintained between 44-47°C. Then, the medium was poured into sterilized Petri dishes and allowed to solidify on a flat cold surface, after which, the plates were inverted to prevent the moisture formed on the plate covers from dropping back on to the solidified medium. Subsequently, the plates were transferred into the incubator where the medium in the plates was dried at 37^0C for 24 hours.

3.3.4 Pig stool and pork meat samples preparation

The deep frozen pig stool and pork samples were carefully removed from the deep freezer and thawed at room temperature. 25g of the both pork muscle and pig feces were weighed and transferred to sterile stomach bags, followed by the addition of 225ml cold buffered peptone water for dissolution and homogenization. Subsequently, the homogenates were packed

in a holding wire mesh box and incubated at 37^0C for 24 hours for pre-enrichment. It should be noted here that, the pork muscle samples were first cut into fine particles by using a scalpel blades in order to allow sequel separation containing *Salmonella* from the pork muscle lamps before dissolving them in buffered peptone water.

3.5 Isolation of *Salmonella* from pork and fecal samples

Specifically, isolation of *Salmonella* was carried out according to the standard protocol described by the International Organization for Standardization 1993 -ISO 6579, the White-Kauffmann-Le Minor scheme and the thin agar layer method (ISO, 1993; Kang and Fung, 2000; Guibourdenche *et al*., 2010), with minor modifications. Twenty five grams of pork muscle and 25g pig fecal samples were weighed and transferred to sterile Stomacher bags (Stomacher®, Seward Limited, UK), containing 225ml of cold sterilized peptone water (Thermo Scientific, Oxoid, UK). This was followed by homogenization using a pulsefying machine (Stomacher® 400 Circulator, Thermo Fisher scientific, UK). The homogenates were incubated at 37^0C for 24 hours for pre-enrichment. Broth was added to the 1 ml bug to make a 5 ml final volume in 5 ml of tetrathionate broth (Thermo Scientific, Oxoid microbiology products, UK) and incubated at 37°C for 24 hours. Xylose Lysine Desoxycholate (XLD) agar (Thermo Scientific, Oxoid microbiology products, UK), was used as selective medium to isolate *Salmonella* from the enriched samples. Plates were incubated at 37^0C overnight, observed for *Salmonella* colony forming units (CFU) and detected according to the protocol described by (CLSI, 2010).

3.6 Confirmation step using biochemical tests

3.6.1 Indole test

Five ml of Indole broth medium was sterilized by autoclaving at 121^0C for 1 hour in the test tubes. The sterilized medium was allowed to cool at room temperature for 1 hour. Using a sterile inoculation loop, a single colony of *Salmonella* isolate was inoculated into the medium, covered with sterile gauze, incubated at 37^0C for 48 hours and observed for colour changes.

3.6.2 Triple Sugar Iron (TSI) test

Five ml of TSI agar medium was sterilized by autoclaving at 121^0C for 1 hour in clear 10 ml vials. The sterilized medium was allowed to cool at room temperature for 1 hour. The caps were aseptically loosened and using a sterile inoculation loop, a single colony of *Salmonella*

isolate was stabbed into the middle of the medium, the caps were tightened, incubated at 37^0C for 48 hours and observed for colour changes.

3.6.3 Simon Citrate test

Five ml of Simon Citrate agar medium was sterilized by autoclaving at 121^0C for 1 hour in clear 10 ml vials. The sterilized medium was allowed to cool at room temperature for 1 hour. The caps were aseptically loosened and using a sterile inoculation loop, a single colony of *Salmonella* isolate was stabbed into the middle of the medium, the caps were tightened, incubated at 37^0C for 48 hours and observed for colour changes.

3.6.4 Urease test

Five ml of Urea agar medium was sterilized by autoclaving at 121^0C for 1 hour in clear 10 ml vials. The sterilized medium was allowed to cool at room temperature for 1 hour. The caps were aseptically loosened and using a sterile inoculation loop, a single colony of *Salmonella* isolate was stabbed into the middle of the medium, the caps were tightened, incubated at 37^0C for 48 hours and observed for colour changes.

3.7 Susceptibility testing by disc diffusion

Susceptibility tests were performed according to Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2010). Antimicrobial minimum disc diffusion inhibitory concentrations (in mms) for test isolates were determined using the Sensititre automated antimicrobial susceptibility system (Oxoid Limited Wade Road Basingstoke Hampshire RG24 8PW, United Kingdom). Interpretation of the results was performed according to the National Committee for Clinical Laboratory Standards (NCCLS), (2003). Antimicrobial susceptibility and resistance pattern of *Salmonella* isolates were examined against including, Ampicillin (10µg), Penicillin (10µg) Tetracycline (10µg) and Sulphamethoxazole (25µg) (Oxoid Limited Wade Road Basingstoke Hampshire RG24 8PW, United Kingdom), antibiotic discs diffusion method (Bauer *et al.,* 1966). Specifically, a suspension of *Salmonella* culture was made at 0.5 McFarland turbidity standard and spread with a sterile cotton swab over the entire surface of Mueller Hinton agar (Oxoid Limited Wade Road Basingstoke Hampshire RG24 8PW, United Kingdom) making a plaque of cells. The antimicrobial discs were applied at specific positions on the plaque surface of Salmonella cells on Muller Hinton plate and incubated at 37^0C for 48 hours. The diffusion radii in millimeters (mm) were measured, recorded and compared with standard measurements in

mm (CLSI, 2010) in order to identify resistant *Salmonella* isolates as defined by ISO protocol (ISO, 1993).

3.8 Molecular biology analysis

3.8.1 DNA extraction from bacterial cells

The 54 *Salmonella* isolates that were confirmed positive by biochemical tests, were further characterized for antimicrobial resistance genes. *Salmonella* Genomic DNA was isolated using Invitrogen PureLink Genomic DNA extraction (Invitrogen) kit according to the manufacturer's instructions. Briefly, bacterial cell cultures in Trypticase Soy Broth (TSB) (Oxoid Limited Wade Road Basingstoke Hampshire RG24 8PW, United Kingdom) were pelleted and harvested by centrifugation at 14000 rpm for 1 min. The supernatant was discarded and the bacterial cell pellets were resuspended in 180µl of Genomic (nuclei) Lysis Buffer Solution (Invitrogen). The bacterial cell pellet suspension was mixed well in Genomic Lysis Buffer by brief vortexing to yield a homogeneous solution. 20µl of Proteinase K was added to the homogenate suspension in order to precipitate the proteins in the bacterial cells pellet suspension followed by vortexing for 20 seconds before incubating the yielded homogenate at 55°C for 60 mins. To precipitate and remove RNA, 20µl of RNAse was added to the suspension and mixed well by brief vortexing followed by incubation at room temperature for 2 mins. Then, 200µl of Genomic Binding Buffer (Invitrogen) containing isopropanol was added, mixed well and allowed to precipitate DNA. Then, 200µl of 70% ethanol (ice-cold) was added to the suspension mixed well by vortexing in order to wash and harvest the pure DNA. Then approximately, 640µl of the suspension were transferred to sterile Spin Columns in clean Collection Tubes and centrifuged at 13,000rpm for 1 min. The flow through was discarded and the Spin Columns placed in new clean Collection Tubes followed by the addition of 500 µl of Wash Buffer 1(Invitrogen), vortexed briefly and centrifuged at 13000rpm for 1 min. The flow through was discarded and the Spin Columns transferred into new clean Collection Tubes followed by the addition of 500µl of Wash Buffer 2 and centrifuged at 13000rpm for 1 min. The flow through was once again discarded and the Spin Columns transferred to clean Collection Tubes and centrifuged at 13000rpm for 1 min. Further centrifugation was carried out to remove traces of ethanol which is normally an unnecessary inhibitor of subsequent DNA manipulations including PCR amplification. The Spin Columns were transferred to sterile 1.5ml eppendorf tubes followed by the addition of 50µl nuclease free water for the elution of the pure bacterial genomic DNA by centrifugation at 14,000rpm for 1mins. The Spin Columns were kept under refrigeration for a second elution using 25µl of

nuclease free water. The eluted Ultra-pure DNA recovered was appropriately stored at 4° C for short term and at -20° C for long term storage.

3.8.2 Determination of the quality and quantity of bacterial DNA

The quality of the extracted DNA was measured using the Nanodrop 2000/2000cc spectrophotometer (BioRad, biotin). The absorbance at ratio at 260/280 was recorded as an indicator of the quality of DNA. Further DNA quality assurance analysis was carried out by gel electrophoresis using lambda DNA as a control molecular marker (ladder). Visualization was carried out by UV light trans-illumination after staining the DNA using gel red.

3.8.3 Analysis of antimicrobial resistance genes using PCR

Salmonella positive isolates were analyzed for the presence of *tetA (A), tetA (B), tetC (A), Sul1, Sul2, Sul3* and *blaCTX-M2* antimicrobial resistance encoding genes using specific primers (table 3.1). Primary PCR was performed according to the protocol described by (Zankari *et al.,* 2012), with slight modification in order to detect different gene product targets. Briefly, the PCR was carried out using the Bioneer PCR, PreMix Kit (Accupower PCR premix, Cat No. 2012K) and according to the manufacturer's instructions. The PCR reaction was carried out in a 20 µl strip consisting of 0.5 µl forward primer, 0.5 µl reverse primer, 1.0µl genomic bacterial DNA and 18.0 µl nuclease free ddH_2O. The Bioneer Kit (ready to use) contained the right proportions (in a single pellet) of the PCR buffer (10mM Tris-HCl, pH 9.0; 1.5mM $MgCl_2$; 30mM KCl; Stabilizer and tracking dye), 250 µM each of dATP, dGTP, dCTP, dTTP (Bioneer Corperation) and 1.0U Top DNA Taq polymerase. The PCR mixture was centrifuged for 1 min at 2500 rpm using the Rottar Centrifuge (BeckMan Coulter Allegra^{TM} 25R Centrifuge) in order to spin down the mixture droplets. Then the PCR mixture in strips was loaded into PCR Thermocycler (Gene-Amp PCR System 9700- Applied BioSysetms). The PCR conditions were set as follows; an initial denaturation at 95℃ for 2 min followed by 35 cycles of 95℃, 30s; 55℃, 30s; 72℃, 30s, and a single final extension of 10 min at 72℃. The amplicons were analyzed by electrophoresis on 1.8 % agarose gels which were stained with gel red (Biotium, Biotium Corporate Headquarters, Biotiuminc, Hayward, CA) and visualized by UV trans-illumination.

Table 3.1. List of Oligonucleotide primers used for PCR amplification of antimicrobial resistance genes.

gene	Primer	PCR primer sequence (5', 3')	Position	Size	Tm	Acc. No.
tetA (A)	tetA(A)-F	GCTACATCCTGCTTGCCTTC	7165-7278	831	55	S52437
	tetA(A)-R	CATAGATCGCCGTGAAGAGG	7562-7581	831	55	
tetB (B)	*tetB* (B)-F	CCCAGTGCTGTTGTTGTCAT	2003-2022	723	55	V00611
	tetB(B)-R	CCACCACCAGCCAATAAAAT	2661-2642	723	55	
tetC (A)	tetC(C)-F	TCAACCCAGTCAGCTCCTTC	675-694	1019	55	J01740
	tetC(C)-R	GAGCACATGGAACGGGTT	1192-1174	1019	55	
Sul1	S1-F	GTGACGGTGTTCGGCATTCT	1738-1758	400	55	M73819
	S1-R	TTTACAGGAAGGCCAACGGT	2406-2387	400	55	
Sul2	S2-F	TTTTCGGCATCGTCAACATA	7894-7914	400	55	M28829
	S2-R	CAATGCTCTGCAGCGAGTGT	8579-8568	400	55	
Sul3	S3-F	AAGTGGGCGTTGTGGAAGA	300-320	400	55	AY494779
	S3-R	CTGACTTTGCCAAGCCTGAA	353-333	400	55	
blaCTX-M2	bla-F	GGCGTTGCGCTGATTAACAC	43490-43509	486	55	X92507
	bla-R	TTGCCCTTAAGCCACGTCAC	44079-44060	486	55	

List of primers used for specific amplification of tetracycline, sulfamethoxazole and beta-lactamase target genes in *Salmonella* isolates (Zankari *et al.,* 2012).

3.8.4 PCR analysis of housekeeping genes

Polymerase chain reaction analysis was carried out targeting the following seven housekeeping genes, *aroC* (chorismate synthase), *dnaN* (DNA polymerase III beta subunit), *hemD* (uroporphyrinogen III cosynthase), *hisD* (histidinol dehydrogenase), *purE* (phosphoribosylaminoimidazole carboxylase), *sucA* (alpha ketoglutarate dehydrogenase) and *thrA* (aspartokinase+homoserine dehydrogenase). Gene amplifications were performed using Bioneer Premix Kit (Accupower® PCR Premix. Bioneer Corporation, South Korea). 20µl reaction mixture consisting of 0.5 µl - forward primer at 10 µM, 0.5 µl – reverse primer at 10 µM, 1 µl

DNA template at 20ng/µl, 18 µl ddH_2O nuclease free and a pellet consisting of Top DNA polymerase 1U, each dNTP (dATP, dCTP, dGTP and dTTP) 250 µM, Tris-HCl (pH 9.0) 10mM, KCl 30mM, $MgCl_2$ 1.5mM, stabilizers and tracking dye was used. The PCR mixture was loaded into PCR Thermocycler (Gene-Amp PCR System 9700- Applied Biosystems, UK) with the following conditions: an initial denaturation at 95℃ for 2 min followed by 35 cycles of 95℃, 30s; 55℃, 30s; 72℃, 30s, and a single final extension of 10 min at 72℃. Table 3.2 shows the list of primers used to perform the primary PCR.

Table 3. 2. List of Oligonucleotide primers for PCR analysis of seven housekeeping genes

Gene	PCR pair primer sequence (5', 3')	Product Size (bp)	Tm (0C)
thrA			55
	F 5'-GTCACGGTGATCGATCCGGT-3'	852	
	R 5'-CACGATATTGATATTAGCCCG-3'		55
		510	
purE	F 5'-ATGTCTTCCCGCAATAATCC-3'		55
		643	
	R 5'-TCATAGCGTCCCCCGCGGATC-3'		55
		894	
sucA			55
	F 5'-AGCACCGAAGAGAAACGCTG-3'	826	
	R5'-GGTTGTTGATAACGATACGTAC-3'		55
		666	
hisD	F 5'-GAAACGTTCCATTCCGCGCAGAC-3'		55
		833	
	R 5'-CTGAACGGTCATCCGTTTCTG-3'		55
		852	
aroC	F 5'-CCTGGCACCTCGCGCTATAC-3'		55
		510	
	R 5'-CCACACACGGATCGTGGCG-3'		55
		643	
hemD	F 5'-ATGAGTATTCTGATCACCCG-3'		55
		894	
	R 5'-ATCAGCGACCTTAATATCTTGCCA-3'		55
		826	

dnaN			55
	F 5'-ATGAAATTTACCGTTGAACGTGA-3'	666	
	R 5'-AATTTCTCATTCGAGAGGATTGC-3'	833	55

List of primers which were used for specific amplification of the seven housekeeping genes in *Salmonella* isolates (MLST website at http://mlst.ucc.ie- University of Cork, UK, 2013).

3.8.5 Sequencing of PCR products

The PCR products were purified with High PCR Purification kits (Qiagen kit, UK) and sequenced at the Segolip sequencing Unit at BecA-ILRI Hub using ABI 3730 Genetic Analyzer (Applied Biosystems, UK). Resultant DNA sequence data were compared to data in the GenBank database using the BLAST algorithm (Korf *et al*., 2003) available at the National Center of Biotechnology Information's web site (www.ncbi.nlm.nih.gov). The Sequences were assembled using CLC Genomics Workbench downloaded from http://www.clcbio.com. BLASTn was performed according to the protocol by (Korf *et al*., 2003), at the publicly available geneBank database at www.ncbi.nlm.nih.gov website in order to identify the isolates. Table 3.3 lists the primers which were used to perform PCR sequencing.

Table 3.3. Oligonucleotide primers used for PCR sequencing analysis of the seven housekeeping genes

Gene	***Primer name***	**Primer sequence (5', 3')**	**Tm (0C)**
thrA	**F**	5'-ATCCCGGCCGATCACATGAT-3'	55
	R	5'-CTCCAGCAGCCCCTCTTTCAG-3'	55
purE	**F**	5'-CGCATTATTCCGGCGCGTGT-3'	55
	R	5'-CGCGGATCGGGATTTTCCAG-3'	55
sucA	**F**	5'-AGCACCGAAGAGAAACGCTG-3'	55
	R	5'-GGTTGTTGATAACGATACGTAC-3'	55
***hisD*:**	**F**	5'-GTCGGTCTGTATATTCCCGG-3'	55
	R	5'-GGTAATCGCATCCACCAAATC-3'	55
aroC	**F**	5'-GGCACCAGTATTGGCCTGCT-3'	55
	R	5'-CATATGCGCCACAATGTGTTG-3'	55
hemD	**F**	5'-GTGGCCTGGAGTTTTCCACT-3'	55

	R	5'-GACCAATAGCCGACAGCGTAG-3'	55
dnaN	**F**	5'-CCGATTCTCGGTAACCTGCT-3'	55
	R	5'-CCATCCACCAGCTTCGAGGT-3'	55

List of primers which were used for sequencing the PCR products (MLST website at http://mlst.ucc.ie- University of Cork, UK, 2013).

3.8.6 Multi-locus sequence type (MLST) analysis and assignment of Sequence types (STs)

MLST was performed according to the protocol described by (Harbottle *et al*., 2006; Martinez-Murcia *et al*., 2011). The sequences were concatenated and aligned using ClustalW (Thompson *et al*., 1994) algorithm in MEGA4.0 (Tamura *et al*., 2007). The complete nucleotide sequences of the seven housekeeping gene loci in the following order (*aroC,dnaN, hemD, hisD, purE, sucA and thrA*), were used to query the MLST website. Interrogation of the *Salmonella* MLST website automatically assigned existing or new allele type (sequence type (ST)) numbers as defined by the database. Composite sequence types (STs) were assigned based on the set of allele types derived from each of the seven loci. The sequence types (STs) obtained were used for reconstructing phylogenetic trees in MEGA 4.0 (Tamura, *et al*., 2004) using the neighbor- joining method algorithm (Nei and Gojobori, 1986; Tamura, 2004). *Salmonella enterica subsp. enterica* serotype Typhimiurium, strain VNP20009, accession number CP007804.2 was downloaded from the www.ncbi.nlm.nih.gov database website and used as a reference serotype. The new STs were submitted to the MLST database website.

3.8.7 Taxonomic cluster analysis

Sequences were aligned using ClusalW (Thompson *et al*., 1994) and compared to *Salmonella* housekeeping reference gene sequences downloaded from the MLST database. A cluster analysis was performed with the Dice coefficient using the Unweighted Pair Group Method with arithmetic Averages (UPGMA dendrogram). Cluster analysis was used to define different groups of serotypes. The phylogeny algorithms in MEGA4.0 (Tamura *et al*., 2007) were used to construct the phylogenetic tree and the evolutionary history of the isolates was inferred by using UPMGA.

3.9 Data management and analysis

Raw data was initially obtained and recorded in a hard cover notebook. This was followed by placing data into rows and columns in a table format for further analysis. *Salmonella* isolates were examined and confirmed by biochemical tests. Numbers positive were presented in percentages (table 4.1). Biochemical analysis revealed colour changes in different media tests (table 4.2). Confirmed *Salmonella* isolates were subcultured in XLD where typical round colonies of *Salmonella* were displayed (figure 4.1). Susceptibility examination revealed different minimum inhibition diameters in mm which were converted to percentages (table 2). Antimicrobial resistance gene analysis was revealed by gel electrophoresis (figure 4.2 and figure 4.3). Similarly, gel electrophoresis of PCR products of the seven housekeeping genes was revealed (figure 4.4). Briefly, the sequence data were unzipped using (CLC workbench), edited, gaps removed, conflicts resolved and assembled into contigs using predetermined statistical algorithms in the software (O'Neil *et al*., 2014; Perceptual Edge-Jonathan Koomey, 2006). The contigs assembled were converted into FASTA format (appendix 2) and aligned at publicly accessible www.ncbi.nlm.nih.gov website to identify *Salmonella*. FASTA sequences were converted into MEGA sequence format (appendix 3) and aligned using CLUSTALw. Predetermined and build-in algorithms in MEGA were used for sequence type analysis of six taxa (figure 4.5), overall phylogenetic analysis (figure 4.6) and for identifying changes due to selection between isolate sequences (table 4.6 and table 4.7).

3.9.1 Modeling and algorithms

Mathematical formulas or models called algorithms were applied for sequence data analysis in order to identify relationships among the isolate sequence. The variables were applied to determine sequence type distances, codon based test of neutrality between sequences and estimate the pattern of nucleotide substitutions. In general terms, such models have been developed to evaluate a particular variable in the data based on other variable(s) in the data, with some residual error depending on model accuracy (i.e., Data = Model + Error) (Judd *et al*., 1989). The objective was to determine the criteria for rejecting the null hypothesis, that, sequences evolve with the same pattern of substitution as judged from the extent of differences in base composition between sequences as measured by the MEGA 4 algorithm (Tamura *et al*., 2007).

3.9.1.1 Codon-based Test of Neutrality for analysis between sequences

This test was used to estimate the probability of rejecting the null hypothesis by considering strict-neutrality ($d_N = d_S$) between sequences. Values of *P* less than 0.05 were considered sig-

nificant at the 5% level. The test statistic (d_N - d_S) evaluated the d_S and d_N which are the numbers of synonymous and nonsynonymous substitutions per site, respectively. The variance of the difference between the variables was computed using the bootstrap method replicates in which analyses are conducted using the Nei-Gojobori method in MEGA4 (Nei and Gojobori, 1986; Tamura, *et al*., 2007). All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

3.9.1.2 Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

Finally, the Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution (Tamura *et al*., 2004) was performed in order to confidently determine the a criterion for rejecting the null hypothesis. The test is based on the probability of substitution from one base (row) to another base (column) instantaneously as defined by MEGA software algorithms. Only entries within a row are compared. The test yields the rates of different transitional substitutions which are shown in bold and those of transversional substitutions normally shown in italics table 4.5. All positions containing gaps and missing data were eliminated from the dataset (Complete-deletion option). There were a total of 80 positions in the final dataset. All calculations were conducted in MEGA4 (Tamura *et al*., 2007).

CHAPTER FOUR

4.0 RESULTS

4.1 Identification of *Salmonella* by conventional culture

Out of 200 samples (100 fecal and 100 muscle samples) collected and cultured, 54 samples were confirmed to be positive for *Salmonella* (33 fecal and 21 muscle samples respectively) using biochemical tests. This represented 16.5% fecal and 10.5 % muscle samples, yielding an overall 27% *Salmonella* positive isolates (table 4.1).

Table 4.1 Summary of results upon conventional culture isolation of *Salmonella*

Serial/No.	Type of Sample	Sample Size	No. Positive samples	% of positive samples
1	Fecal	100	33	16.5
2	Muscle	100	21	10.5
Total		200	54	27

Table 4.1 numbers and percentages of confirmed *Salmonella* positive isolates.

4.2 biochemical tests for identifying S*almonella* isolates

4.2.1 Indole test

Salmonella positive isolates were correctly identified with the production of a pink ring on the surface of Indole (COVAK'S) broth medium (table 4.2)

4.2.2 Triple Sugar Iron (TSI) test

Black colour change of TSI medium provided evidence for *Salmonella* positive isolates detection (table 4.2).

4.2.3 Simon Citrate test

Colour change of Simon Citrate agar medium from green to deep blue correctly identified *Salmonella* positive isolates (table 4.2).

4.2.4 Urease test

No colour change in Urea agar medium provided enough negative confirmatory evidence for identifying *Salmonella* (table 4.2).

Table 4.2 confirmatory biochemical tests for identifying *Salmonella* isolates

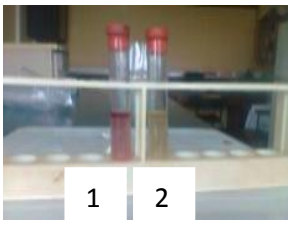
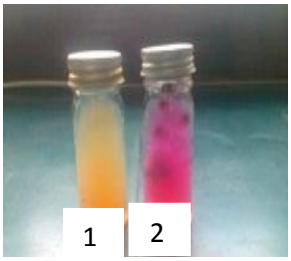
	Test	Colour change	Remarks
1	Indole		Pink ring in 1 no colour change in 2
2	Triple Sugar Iron		Black colour in 1 no colour change in 2
3	Simon Citrate		Blue colour in 1 no colour change in 2
4	Urease		No colour change in 1 colour change in 2

Table 4.2 confirmatory biochemical test performed on *Salmonella* isolates.

Confirmed *Salmonella* positive round colonies with a black center typical of the morphological characteristic features of *Salmonella* on XLD agar plate (figure 4.1).

Figure 4.1 Typical *Salmonella* colonies

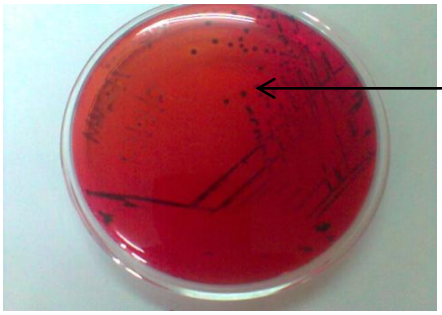


Figure 4.1 *Salmonella* colonies on XLD

4.3 Susceptibility testing

4.3.1 Susceptibility percentages

Susceptibility pattern for tetracycline, ampicillin, penicillin and sulfamethoxazole were found to be 43%, 9%, 56% and 83% respectively. Intermediate susceptibility levels of 17%, 18%, 17% and 17% were recorded for tetracycline, ampicillin, penicillin and sulfamethoxazole respectively. Similarly, the resistance phenotypes were found to be 40%, 73%, 27% and 0% for tetracycline, ampicillin, penicillin and sulfamethoxazole respectively, (table 4.3).

Table 4.3. Percentage (%) susceptibility

Category	Tetracycline	Ampicillin	Penicillin	Sulfamethoxazole
1 Susceptible	43	9	56	83
2 Intermediate	17	18	17	17
3 Resistant	40	73	27	0

The results were compared to the standard disc diffusion breakpoints in mm and converted to percentages.

4.3.2 Phenotype colony susceptibilities

The susceptibility phenotypes revealed different diameters (mm) of inhibition on Nutrient agar plates. In the negative control, no inhibition zones were observed. Inhibition zones were comparable between the test sample and the positive control (table 4.4).

Table 4.4 Representative disc diffusion plates

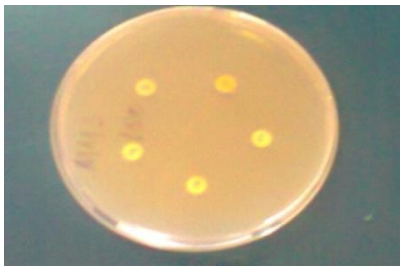
Serial No.	Test	Susceptibility test (mm)	Remarks
1	Negative control		No inhibition observed after incubation at 37^0C overnight.
2	Positive control		Inhibition observed after incubation at 37^0C overnight.
3	Test sample		Inhibition observed after incubation at 37^0C overnight.

Table 4.4 Susceptibility disc inhibition zones of tetracycline, ampicillin, penicillin and sulfamethoxazole.

4.4 Molecular analysis of the confirmed *Salmonella* positive isolates

4.4.1 Beta-lactamase resistance gene analysis

Isolates carrying *blaCTX-M2* were correctly detected by PCR as being equally distributed among all the tested isolates. The expected band size of 450 base pairs (bp) was correctly amplified using specific primers. Sample 33, yielded no band, while there were two products in samples 3, 15 and 28. This can be explained as a result of unspecific amplification attributed to lack of optimized conditions such as temperature optimization (figure 4.2).

Figure 4.2 ^{bla}*CTX-M2* **gene fragment size**

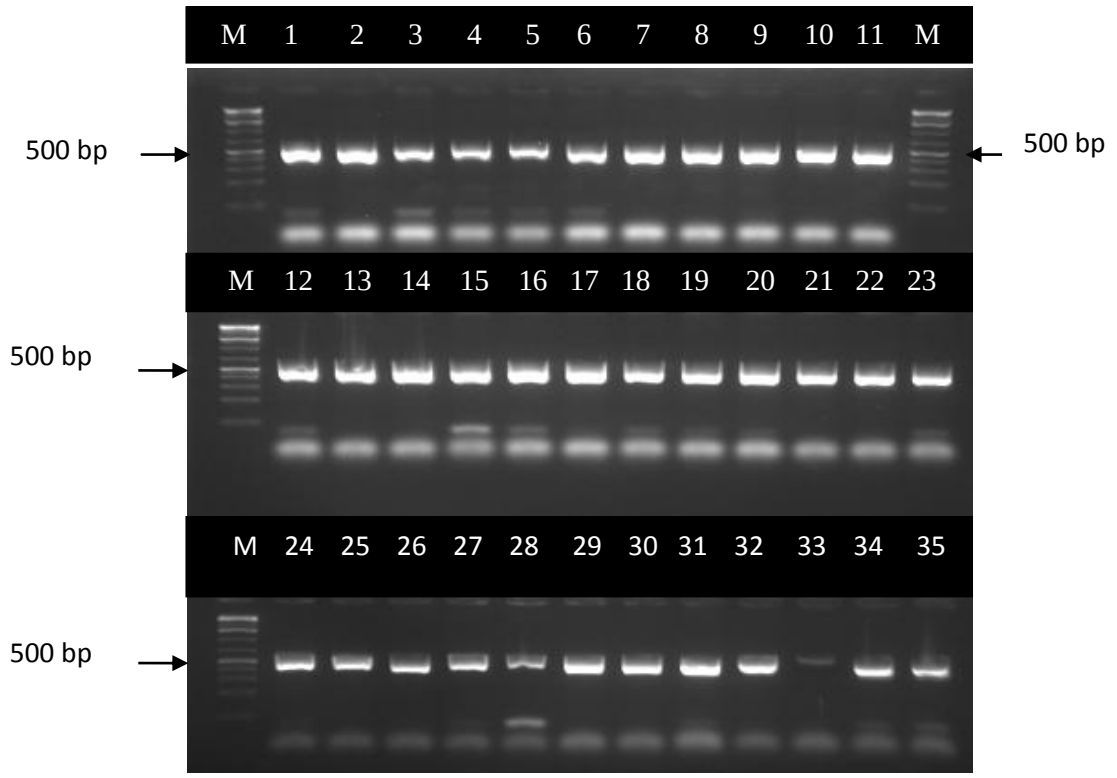


Figure 4.2 Gel photo of ^{bla}*CTX-M2* gene fragment. M is 1 KB+, a molecular marker, lanes 2-11, 12-23 and 24-35 were the samples of *Salmonella* isolates analyzed by gel electrophoresis. In lane 33 the band formed was not clear while in lanes 15 and 28 small bands were seen next to the primer dimmers.

4.4.2 Sulfonamide resistance gene analysis

None of the sulfonamide resistance gene markers including *sul1, sul2* and *sul3* were detected upon PCR analysis.

4.4.3 Tetracycline resistance gene analysis

Amongst the *tet* genes evaluated, only *tetB(B) gene* was detected in 80% of the isolates. The expected band size of 723 base pairs was correctly amplified using specific primers (figure 4.3).

Figure 4.3 *tetB(B)* gene amplicon size

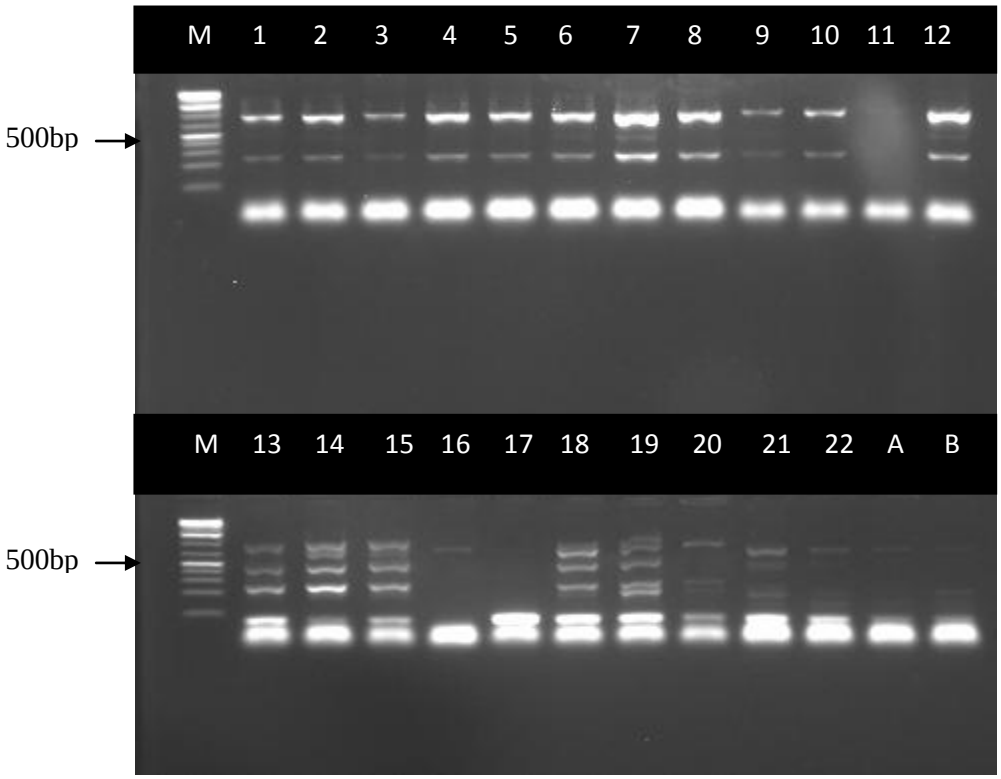


Figure 4.3 Gel photo of *tetB(B)* gene fragment. M is 1KB+, a molecular marker, 2-12 and 13-22 DNA samples of *Salmonella* isolates analyzed by gel electrophoresis. 11 was a smear which occurred due to shearing of DNA, 16, 16 and 22 formed no clear band. A and B were used as the negative controls.

4.4.4 PCR product analysis of the housekeeping gene

The seven housekeeping genes evaluated using specific primers were correctly amplified. The expected fragment sizes corresponding to each of the seven housekeeping genes were confirmed by gel electrophoresis in all the isolates. The expected band size of the *thrA* gene was 510bp (*Salmonella* MLST database website at the University of Cork, UK, 2013). The representative gel images obtained up on gel electrophoresis of the *thrA gene* (figure 4.4).

Figure 4.4 PCR product of *thrA* gene fragment

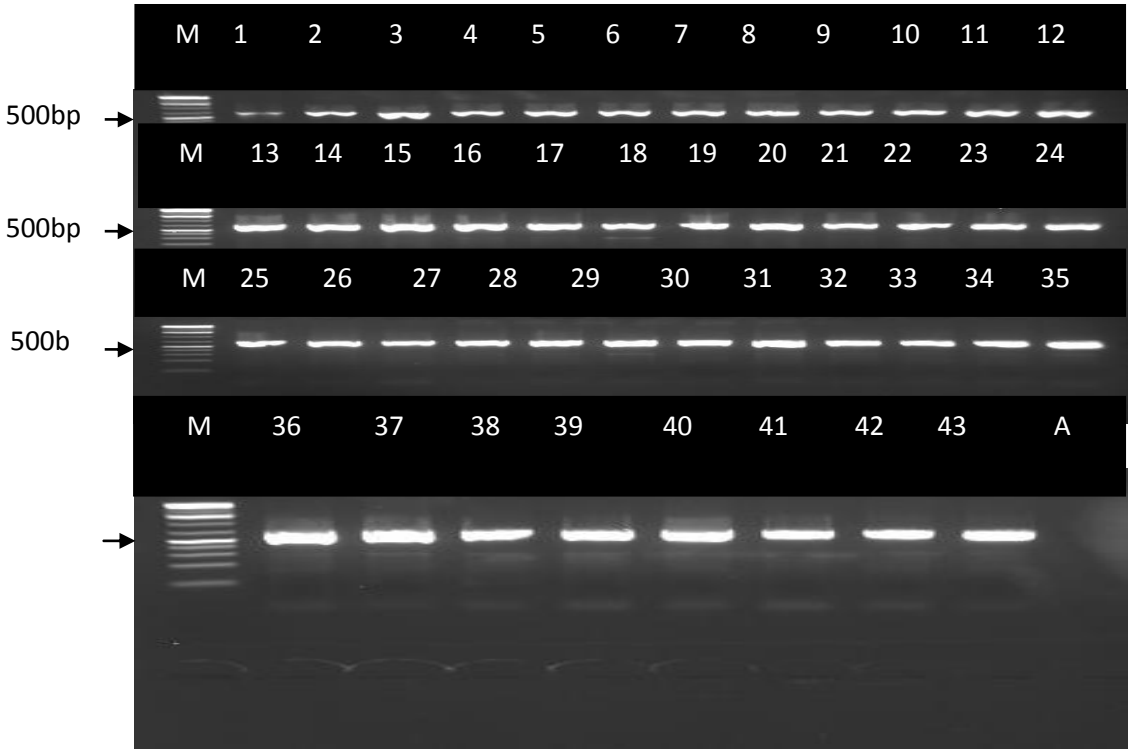


Figure 4.4 Gel photo of *thrA* gene fragment. M is 1KB+, a molecular marker, lanes 1-43 are DNA PCR products of *Salmonella* housekeeping gene fragments analyzed by gel electrophoresis. A is the negative controls containing all the master mix other than the sample PCR product.

4.5 BLASTn at www.ncbi.nlm.nih.gov

BLASTn analysis revealed an E-value of 0.0 and a percentage identity value of between 98%-99% with a query cover of 95%. Accession numbers of *Salmonella* serotypes with which the isolates studied here were found to be identical include, *S.* Paratyphi, *S.* Abony, *S.* Newport, *S.* Bovismorbificans, *S.* Bareilly, *S.* Thompson, *S.* Dublin and *S.* Typhimurium (table 4.5).

Table 4.5 Identification of isolates at www.ncbi.nlm.nih.gov

Report	Description	Max score	Total score	Query cover%	Evalue	Ident%	Accession
1	*S.* Paratyphi	1077	1077	95	0.0	99	CP000886.1
2	*S.* Abony	1077	1077	95	0.0	99	CP007534.1
3	*S.* Newport	1072	1072	95	0.0	99	CP007216.1
4	*S.* Bovismorbificans	1072	1029	95	0.0	99	HF969015.2
5	*S.* Bareilly	1072	1072	95	0.0	99	CP006053.1
6	*S.* Thompson	1066	1066	95	0.0	99	CP006717.1
7	*S.* Dublin	1061	1061	95	0.0	99	LK931502.1
8	*S.* Dublin	1061	1061	95	0.0	99	CP001144.1
9	*S.* Typhimurium	1044	1044	95	0.0	99	CP007235.1
10	*S.* Typhimurium	1044	1044	95	0.0	98	CP009102.0

Table 4.5 serotypes described here were obtained by BLASTn of the FASTA format sequence at www.ncbi.nlm.nih.gov and they are based on the *sucA* housekeeping gene sequences (appendix 2).

4.6 MLST analysis of the sequences

Different sequence types were discovered. Sequence types (STs) related to ST5 lineage was found. The STs obtained belonged to the ST157 complex but, they were not identical to each other. Novel STs were assigned new allele numbers as follows; ST684, ST466, ST1676, ST1229, ST741, ST98, ST725, ST569, ST546, ST429, ST92, ST78, ST747, ST739, ST640, ST945,ST 861, ST86, ST787, ST772, ST694, ST599, ST582, ST534, ST533, ST1776, ST999, ST998, ST997 and ST996. A unique evolutionary tree, drawn to scale base on these sequence types was constructed (table 4.6).

Table 4.6 The summary of the sequence types

MLST allele sequence analysis and assignment of the new allele sequence types								
ST	aroC	dnaN	hemD	hisD	purE	sucA	thrA	ST Complex
Unknown ST	147	378	10	123	10	19	17	NC
ST684	147	13	15	123	15	19	17	157
ST466	147	13	15	123	140	7	17	157
ST1676	147	13	15	123	15	9	17	NC
ST1229	313	13	15	123	15	19	17	157
ST741	2	201	146	9	6	19	17	NC

Table 4.6 These sequence types were automatically generated by the predefined MLST algorithms and allocated new (for unknown STs) or existing sequence types (for known STs). NC=no complex.

4.7 Analysis of Evolutionary relationships based on ST

The tree shows the evolutionary relationships of six (6) selected ST taxa. The genetic differences between *Salmonella* isolates STs and a reference ST isolate were found. The isolates were clearly differentiated into two clusters 1 and 2. Cluster 1 consists of ST19, ST147 and ST17. Cluster 2 consists of ST 123 and ST 378. *S.* Typhimurium (ST446) was used as a reference ST. It was revealed that, the genetic distances between the isolates in cluster 1 relative to the reference ST were negligible. This implies that these isolates were more similar to each other and to the reference ST. The genetic distances between isolates in cluster 2 were found to be protracted, an indication that these isolates were different from each other and from the reference ST (figure 4.5).

Figure. 4.5 Evolutionary relationships of 6 ST taxa

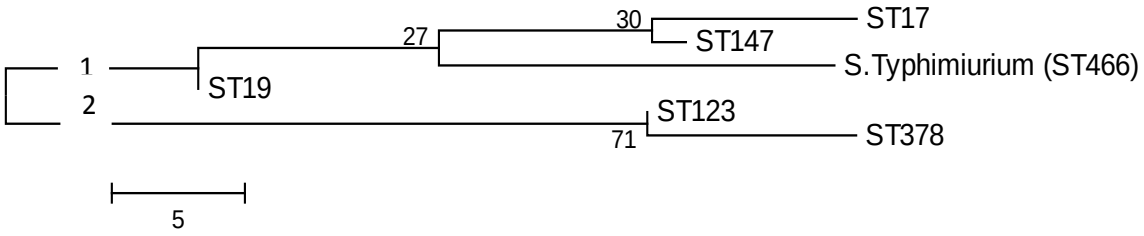


Figure 4.5 Dendogram showing two clusters 1 and 2. Cluster 1 consists of ST19, ST147 and ST17. Cluster 2 consists of ST123 and ST378. *S.* Typhimiurium (ST466) is the reference.

4.8 Codon-based Test of Neutrality for analysis between sequences

This test statistic yielded different probability levels between the sequences. Probability levels greater than 0.05 indicates the highest level of neutrality (similarity) between sequences. On the other hand, probability levels less than 0.05 including 0.0366 in column two, 0.046 in column four and 0.021 in column five indicates lack of neutrality. Similar to MLST analysis ST based evolutionary taxonomic tree (figure 4.5), these results pointed to differences between isolate sequences and the reference sequence (table 4.7).

Table 4.7 Codon-based Test of Neutrality for analysis between sequences

	1	2	3	4	5	6
[1] *S*.Typhimiurium_(ST466)		0.137	-0.877	-0.0618	-0.386	1.726
[2] ST123	0.891		0.762	2.114	0.878	0.910
[3] ST147	0.382	0.448		-0.233	-1.207	-0.0467
[4] ST17	0.951	0.0366	0.816		2.00813	1.569
[5] ST19	0.700	0.382	0.300	0.0469		-2.344
[6] ST378	0.0868	0.364	0.963	0.119	0.0207	

Table 4.7 shows the test statistic (d_N - d_S) above the diagonal. d_S and d_N are the numbers of synonymous and nonsynonymous substitutions per site, respectively. *P* Values of less than 0.05 at the 5% level are highlighted.

4.9 Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

The nucleotide frequencies of 0.177 (A), 0.244 (T/U), 0.263 (C), and 0.317 (G) were obtained. The transition/transversion rate ratios were found to be k_1 = 2.698 (purines) and k_2 = 20.089 (pyrimidines). The overall transition/transversion bias was R = 6.565, where R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]. The results further showed that the sequences types obtained by MLST analysis were genetically diverse (table 4.8)

Table 4.8 Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

	A	T	C	G
A	-	*1.81*	*1.94*	**6.33**
T	*1.31*	-	**39.06**	*2.35*
C	*1.31*	**36.27**	-	*2.35*
G	**3.54**	*1.81*	*1.94*	-

Table 4.8 shows the transversion/transition rate ratios as a measure of sequences divergence

4.10 Assignment of isolate to serotypes taxonomy

The small bootstrap values indicate that the genetic distances between the isolates with a given cluster are negligible. The large bootstrap values conversely indicate that the genetic distance between evaluated isolate sequences are protracted. These are important indicators of genetic divergence between closely related isolates. The isolates differentiated into two main clusters 1 and 2 (figure 4.6).

Figure 4.6 Assignment of isolate to serotypes taxonomy

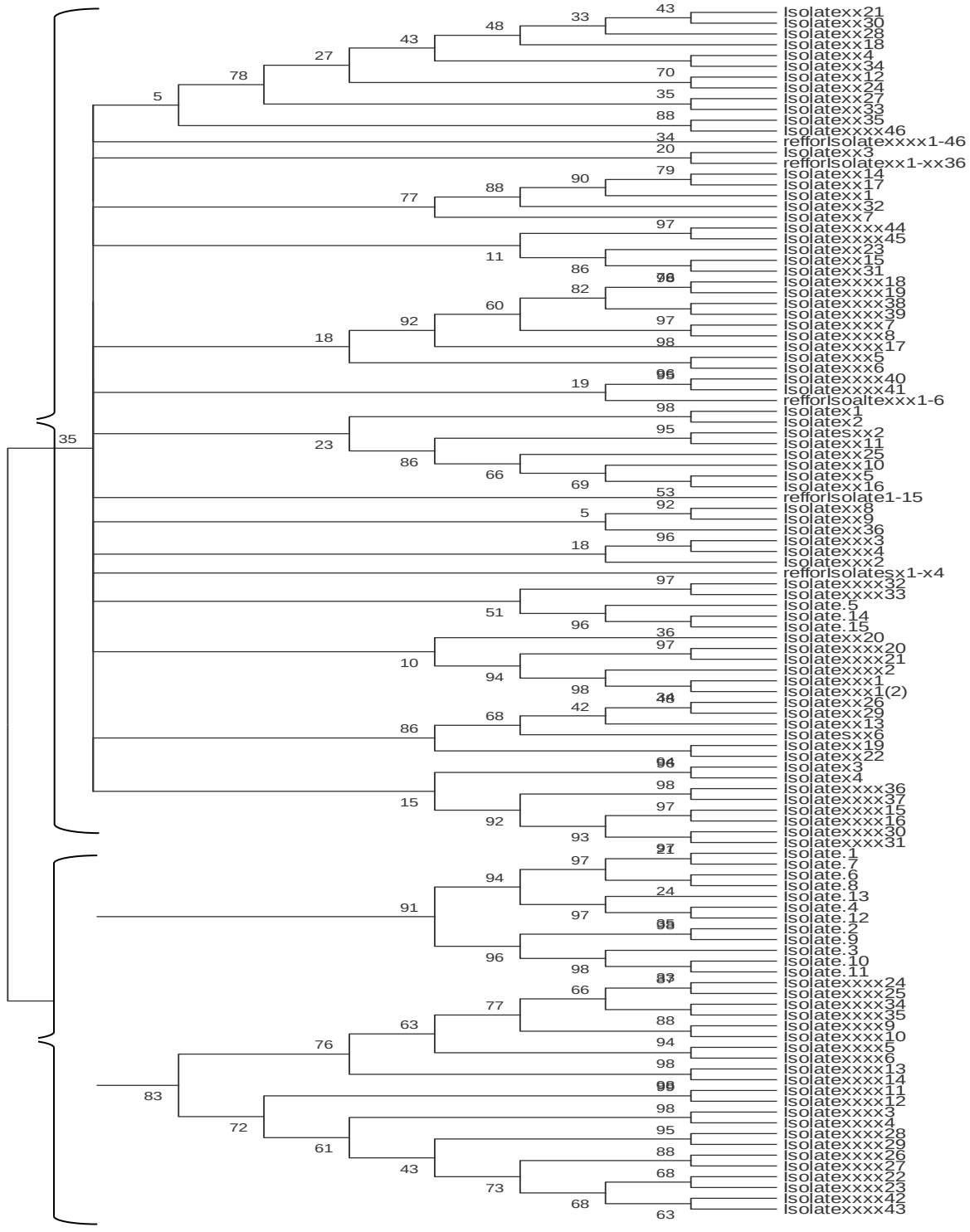


Figure 4.6 The dendogram showing two clusters 1 and 2. The tree was constructed using Unweighted Pair Group Method using arithmetic Averages (UPGMA) algorithm. The results further confirm that the isolates studied here were indeed divergent.

CHAPTER FIVE

5.0 DISCUSSION

Worldwide, salmonellosis is one of the most important foodborne diseases of animals and man (Bean *et al*., 1996). Salmonellosis is one of the pathogens which have been seen to represent an increasing emergence, whose origins are associated with livestock production and food contamination (Aarestrup, 1999). *Salmonella* infections in man and animals are usually caused by handling or consuming contaminated food products (Redmond and Griffith, 2003). Similar to other report by (Guibourdenche *et al*., 2010) using the White-Kauffmann-Le Minor scheme, the isolates studied in this project were identified to be *Salmonella*. Conventional culture methods are important in categorically identifying a microbial pathogen as the sole cause of infection or food contamination (Guibourdenche *et al*., 2010). Antimicrobial resistant isolates were also identified by disc diffusion method, according to the standard ISO protocol and the CLSI guidelines (ISO, 1993; CLSI, 2010). Different disc inhibition radii phenotypes were revealed against tetracycline, ampicillin, penicillin and sulfamethoxazole. Determining antimicrobial susceptibility levels of microorganisms is important in identifying suitable antimicrobial drugs which could be used for adequate treatment of livestock or human patients (CDC, 2005). Accordingly, *S. enterica* species has been recognized to be one of the most important zoonotic food-borne pathogen (Schneider, 2011) and therefore deserves more attention for antimicrobial surveillance. Detection of the genetic determinants of resistance including $bla_{\text{CTX-M2}}$ and *tetA(B)* using PCR analysis appeared to be a valuable alternative method for examining antimicrobial resistance of this pathogen that is of significant public and animal health concern. The results uncovered 100% $bla_{\text{CTX-M2}}$ positive serotypes in the pig samples. These results are consistent with a study performed on French *Salmonella* Typhimurium isolates to determine bla_{TEM} emergence in human and non-human sources which revealed the presence of bla_{TEM} in 26% of human isolates and 23% of animal isolates (Casin *et al*., 1999). It has been reported that, beta-lactamase enzyme production in *Salmonella* continues to be the leading cause of resistance to beta-lactam antibiotics among these gram-negative bacteria (Weill *et al*., 2006). Furthermore, there have been reports of an increasing incidence and prevalence of extended-spectrum beta-lactamases (ESBLs) in recent years (Weill *et al*., 2006). According to (Weill *et al*., 2006), the first ESBLs arose in the early

1980s due to widespread use of broad-spectrum beta-lactamases enzyme producing antimicrobial drugs in man and livestock. The frequency of $bla_{\text{CTX-M2}}$ resistance in *Salmonella* therefore continues to create a major public health anxiety (Weill *et al*., 2006; Chu and Chiu, 2006). While investigating the causes for increasing virulence (Carattoli, 2003), associated the evolution of virulent non-typhoid salmonellosis with antimicrobial resistance plasmids in *Salmonella* species. (Molla *et al*., 2003) identified antimicrobial resistance determinants in *Salmonella* isolates obtained from meat samples of livestock origin in Ethiopia. Findings from our study revealed that *Salmonella* collections from the pigs slaughtered at a centralized abattoir in Uganda indeed carried genetic markers for various antimicrobial agents used for treatment of salmonellosis in pigs.

None of the *Sul* genes tested by PCR were detected in any of the isolates collections tested. This phenomenon is an indication of the presence of an SGI1 variant (Huehn *et al*., 2009) in which molecular determinants could not be detected.

The genes responsible for tetracycline resistance were detected in 80% of the isolates studied during this research. Similar results were obtained in a study by (Kalule *et al*., 2012) who investigated antimicrobial drug resistance and plasmid profiles of *Salmonella* isolates from humans and foods of animals in Uganda. It has been argued that, pathogenic bacterial gain or loses essential regions of their core genome leading to the accumulation of several genetic factors such as pathogenicity islands, expressed sequence types (STs) and modified proteins consequently, making them resistant to antimicrobial drugs (Grimont and Weill, 2007). These changes certainly results to genome restructuring by either natural selection or horizontal transfer of genes important in antimicrobial resistance (Grimont and Weill, 2007). Antimicrobial resistance is related to acquisition of various genetic determinants (Su *et al*., 2004) and that such genetic determinants can be horizontally transferred between individual isolates of different lineages found colonizing different sites of livestock such as the chicken oviducts (Didelot *et al*., 2007; Gantois *et al*., 2009; Didelot *et al*., 2011).

MLST has been used to identify bacterial serotypes (Urwin and Maiden, 2003), identifying STs which belong to various ST complexes and more specifically linking STs to pandemics (Gantois *et al*., 2009). Using MLST, the genetic distance between two closely related strains can be quantitatively estimated as allelic differences in the nucleotide sequences of housekeeping or virulent genes among bacterial strains (Urwin and Maiden, 2003). In this study, ST analysis revealed that the isolates studied were related to ST5 lineage which was found to belong to ST157 complex. Sequence type complexes have been shown to be capable of not only introducing fitness changes in bacteria that enable them to evade the immune system of

higher animals but also enable the bacteria to select for antimicrobial resistance genes and that such lineages are likely to initiate pandemics (Zhang *et al*., 1997; Gantois *et al*., 2009). Gantois *et al*. (2009) showed that, *S*. Enteritidis belonging to ST 11 lineage was found to colonize and persist in chicken oviduct leading to chicken salmonellosis pandemic of between 1980s and 1990s in Japan. According to (Falush *et al*., 2003; Gantois *et al*., 2009), the persistence of ST 11 depends on factors such as fimbriae, flagellae, lipopolysaccharide, cell-wall structure and stress tolerance. Similar to the observation by (Gantois *et al*., 2009), the ST5 belonging to ST157 complex was identified in this study is likely to initiate a pandemic in Ugandan pigs and this may possibly lead to human infections. The ST5 lineage identified in this study contributes to increasing pathogenicity and virulent genes which leads to persistence and diarrheic *Salmonella* serotypes which are perhaps the major causes of diarrhea in pigs in Uganda. (Zhou *et al*., 2013; Leekitcharoenphon *et al*., 2013) using genomics and MLST analysis demonstrated an increasingly emerging clone of *Salmonella enterica* serovar Thimiurium ST313 from Nigeria and the Democratic republic of Congo. MLST analysis during this study detailed a cluster of clonal relationships and thereby showing similar results to those by (Hauser *et al.,* 2012; Leekitcharoenphon *et al*., 2013). Data analysis using the codon-based test of neutrality for analysis between sequences (Nei and Gojobori, 1986; Tamura *et al*., 2007) yielded to the *P* values of less than 0.05 which were considered significant at the 5% level. Thus, the < 0.05 level or a statistical score of > 0.95 gives a 5% level of confidence in the results. This means that there is less than 5% chance than the result was a coincidence if the experiment was one tailed. The < 0.01, or > 0.99 level, gives only a 1% chance of randomness producing the results. The null hypothesis of this study was that, there is no any genetic diversity between antimicrobial resistant *Salmonella* isolates STs obtained from pigs slaughtered at Wambizzi abattoir in Kampala, Uganda. Because the confidence level, P, was less than 0.05, the null hypothesis was rejected in favor of the alternative hypothesis.

Finally, the maximum composite likelihood estimate of the pattern of nucleotide substitution were found to be 0.177 (A), 0.244 (T/U), 0.263 (C), and 0.317 (G). The transition/transversion rate ratios are k_1 = 2.698 (purines) and k_2 = 20.089 (pyrimidines). The overall transition/transversion bias was found to be R = 6.565, where R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]. These changes consequently confirmed that the isolates studied here were divergent thus formed the basis for rejecting the null hypothesis in favour of the alternative hypothesis.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

By applying standard protocols and Clinical Laboratory Standard International guidelines, *Salmonella* was isolated from Ugandan samples of pig origin as the probable cause of pork contamination and antimicrobial resistance to evaluated antimicrobial agents thereby effectively addressing objective one and two. PCR analysis made it possible to easily explore relevant gene determinants associated with antimicrobial resistance in *Salmonella* isolates of pig origin. Based on the presence or absence of specific gene markers such as *tetA(A), tetA(B), tetC(A), Sul1, Sul2, Sul3 and blaCTX-M2.* In this study 100% beta-lactam resistant genes were detected in all the isolates recovered from pig carcasses. Similarly, the *tetB(B)* genes were identify using specific primers with an overall 80% detection. However, findings from this research revealed that some resistance gene determinants could not be detected, especially the inability to detect the *sul* gene hence addressing the second objective of this study.

Although the high-throughput-based MLST approach has become more popular, these tools are limited by the availability of skilled personnel, require sophisticated equipment and bioinformatics analysis, generally not available in routine surveillance laboratories, MLST analysis revealed ST5 lineage with new allele sequences and ST157 complex based on the seven housekeeping genes. This made it possible to address objective three of the study. ST variations plausibly helped to conclude that the isolates analyzed were indeed different hence pointing to genetic diversity and consequently, rejecting the null hypothesis. In general, this study demonstrates a novel and specific, high-throughput, primary PCR-based method that can be used for preliminary analysis of *Salmonella* intra-serotype diversity. Based on the same principle, the specific PCR system can be enhanced and extended to other pertinent targets and genes according to the pathogens to be studied and serotype identification or increasingly emerging new resistance mechanisms.

6.2 RECOMMENDATIONS

More comprehensive research is needed to investigate the occurrence of antimicrobial resistance towards more antimicrobial agents used in livestock health in Kampala, Uganda. The

application of more sensitive and gold standard molecular analysis methods such as pulse field gel electrophoresis should be evaluated in a separate study in order to recover low level salmonellosis infections which could not be recovered in this study. This study was carried out in Kampala, Uganda. Since zoonotic salmonellosis is a food borne pathogen, I recommend that a similar study be carried out in Kenya. Further bioinformatics analysis should be carried out on the genomic DNA obtained in this study to identify the coding and non-coding DNA fragments in *Salmonella* isolates in order to identify Single Nucleotide Polymorphisms (SNPs). To carry out research on functional genomics of *Salmonella* isolates in Kenya and contribute to the establishment of the Kenyan bioinformatics repository since we currently rely on the websites hosted by other countries.

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APPENDICES

1. MLST-Sequence types data used for six taxa phylogeny

GGCCGGCGGATTGTTTTAGATGATCGGTCGTGCGGCCTTTAGCGCTGACGAGAAA
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2. Sequences used for BLASTn analysis

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dnaN3

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3. MEGA format for Sequences types

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TGGTTCCGACTATTCCGCCGCCGTGC
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